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EFFECT OF METABOLIC DISEASE AND XENOBIOTIC EXPOSURE ON
HEPATIC ATP-BINDING CASSETTE (ABC) DRUG TRANSPORTER
EXPRESSION

BY

AJAY CHOWDARY DONEPUDI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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DOCTOR OF PHILOSOPHY DISSERTATION
OF
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ABSTRACT

Drug transporters are membrane bound proteins, which are involved in facilitating both uptake and efflux of xenobiotics, endogenous compounds and their metabolites in various tissues such as liver, kidney, testis, and brain. Alteration in drug transporters may cause imbalances in endogenous compounds such as bile acids, hormones, and bilirubin. Xenobiotic metabolism is inefficient without drug transporters, and drug transporters are recognized as vital mediators for moving polar compounds across membranes. ATP binding cassette (Abc) transporters are a kind of drug transporters, which are ATP-dependent membrane bound proteins involved in transport of wide variety of compounds. Multiple hepatic conditions can alter drug transporter expression such as obesity, oxidative stress, cytokines, drug-induced liver injury and environmental toxicants. Specifically, drug transporter expression is known to alter during several metabolic conditions. For example, both genetically modified and diet-induced obesity models of rodents displayed altered hepatic drug transporter expression. Previous studies shown not only during metabolic syndrome, but also exposure to xenobiotics such as estradiol and polybrominated diphenyls (PBDs), which are endocrine disrupting chemicals, alter drug transporter expression. The objective of this study is to determine whether certain liver conditions, such as development of fatty liver (i.e. steatosis) and developmental exposure to an environmental endocrine disruptor (i.e. Bisphenol A) alter hepatic Abc drug transporter expression in conjunction with nuclear receptor expression. This study also

aims to show, changes in hepatic Phase II and Abc drug transporter expression during metabolic syndrome alters metabolism and disposition of endocrine disruptor such as Bisphenol A (BPA) This research will provide novel observation and mechanisms by which expression of drug transporters will be affected and regulated. To delineate these aspects whole thesis research was divided into three specific sub studies:

In the first study, which is presented as **Manuscript I** shows changes in hepatic Abc drug transporters, uptake transporters, Phase I enzyme expression, and factor involved in regulation of hepatic Abc transporter expression during development of obesity. This study also shows possible physiological and transcription factors role in regulation of hepatic Abc transporter expression during development of obesity. In this study, hepatic gene expression and physiological factors were analyzed in both C57BL/6 and ob/ob mice at different time points such as week-1, 3, 4 and 8 age to capture physiological and gene expression changes that occur during development of obesity. Correlation between physiological changes and gene expression was performed using canonical correlations. Significant correlations were observed between physiological changes, hormones, and gene expression changes during development of obesity. Correlation between metabolism-related hormones and hepatic gene expression are sex-dependent. Correlation between physiological changes and gene expression indicated metabolism-related hormones might have a role in regulation of hepatic genes involved in drug metabolism and transport.

In second study, which is presented as **Manuscript II** shows changes in hepatic Abc transporter expression with developmental exposure to endocrine disruptor BPA and identifies mechanistic pathways by which BPA exposure causes these effects. In this study, female mice are exposed to either control or BPA or ethinyl estradiol (EE) or phytoestrogen enriched diet. Studies were performed in male pups to analyze gene expression and functional activities of respective genes. Developmental exposure to BPA and EE downregulated hepatic drug transporters and Phase II enzyme expression and activity that are involved in BPA metabolism and excretion, whereas genistein co-administration reversed these changes. Changes observed with developmental exposure of BPA were persistently observed even after cessation of BPA exposure to male pups. Decrease in nuclear factors mRNA expression and binding activity could be partially responsible for downregulation of hepatic Phase II enzymes and drug transporter expression. Further, increase in expression of histone deacetylases (Hdac's) upon BPA exposure could be responsible for decreased transcription factor expression and activity. Our data suggest that developmental BPA and EE exposure may work via similar pathways, and greatly affect the expression of key hepatic genes involved in BPA and hormone metabolism and clearance.

In third study, presented as **Manuscript III** shows changes in hepatic drug metabolism gene expression observed during obesity alters BPA, an endocrine disruptor disposition. This study aim to identify whether changes in Phase II and drug transporter expression alters BPA disposition, as increase

in urinary total BPA levels and BPA exposure in humans are correlated to occurrence of obesity. BPA (10 mg/kg, *i.v.*) was administered; parent and its metabolites were analyzed in bile, plasma and urine sample of lean and obese rats. Along with *in vivo* BPA disposition studies, hepatic glucuronidation and sulfation enzymatic assays were performed to identify whether obesity altered hepatic metabolic processes. Changes in hepatic Phase II and III protein expression in obese rats resulted in altered BPA metabolism and disposition. In obese rats, BPA metabolites specifically BPA-glucuronide levels were increased in urine and decreased in bile compared to lean rats. This altered BPA metabolism and disposition during obesity suggests, in humans detailed evaluation of urinary BPA levels such as ratio of metabolite to parent compound, are needed to correlate BPA exposure to occurrence of obesity.

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To my mom and dad

PREFACE

This dissertation has been written in the manuscript format as directed by the guidelines set by the Graduate School at the University of Rhode Island. Each manuscript follows the format of the journal “Drug Metabolism and Disposition”.

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INTRODUCTION

1. ATP Binding cassette (Abc) drug transporters:

The liver is the major organ for metabolism and clearance; it plays a predominant role in determining the circulating systemic concentrations of many endocrine hormones in the body. In general, the metabolism of endogenous chemicals and xenobiotics involves Phase-I, -II and -III (drug transporters) proteins (Figure 1). Drug transporters are membrane bound proteins, which are involved in facilitating both uptake and efflux of xenobiotics, endogenous compounds and their metabolites in various tissues such as liver, kidney, testis, and brain. Alteration in drug transporters may cause imbalances in endogenous compounds such as bile acids, hormones, and bilirubin. Xenobiotic metabolism is inefficient without drug transporters, and drug transporters are recognized as vital mediators for moving polar compounds across membranes. ATP binding cassette (Abc) transporters are a kind of drug transporters, which are ATP-dependent membrane-bound proteins involved in transport of wide variety of compounds. Multiple hepatic conditions can alter drug transporter expression such as obesity, oxidative stress, cytokines, drug-induced liver injury and environmental toxicants. Drug transporters are involved in several critical liver functions such as absorption and excretion, which determines the distribution and hepatic concentrations of endogenous compounds in the body. Changes in drug transporter expression, especially ABC transporters, can cause imbalances in homeostasis of endogenous compounds in body, which may lead to several pathological

conditions, such as cholestasis, hyperbilirubinemia, drug-induced liver injury (Faber et al., 2003; Geier et al., 2003; Aleksunes et al., 2008; Lecureux et al., 2009). Impairment of drug transporters causes several pathological conditions such as cholestasis and hyperbilirubinemia. Mice lacking certain drug transporters, such as *Abcc4*, are more susceptible for liver injury during certain pathological conditions, such as cholestasis. ABC transporters have seven subfamilies from A to G such as ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG (Dean *et al.*, 2001). ABC transporters are primarily involved in efflux of endogenous and exogenous compounds.

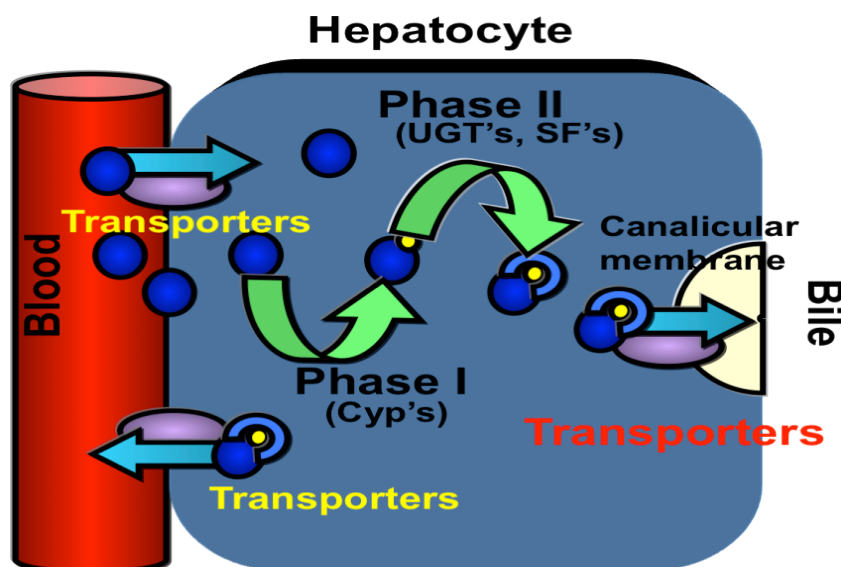


Figure 1 Depiction xenobiotic and endogenous compounds metabolism in liver.

In liver, ABC drug transporters are important in maintaining the bile acid pool, fatty acids, and cholesterol transport and efflux of xenobiotic metabolites. Drug transporters in liver are regulated by several nuclear receptors and transcription factors such as nuclear factor-E2- related factor 2 (Nrf2), aryl

hydrocarbon receptor (Ahr), constitutive androstane receptor (Car), estrogen receptor (Er), and Peroxisome proliferator-activated receptor alpha (Ppar- α) (Faber et al., 2003; Geier et al., 2003; Maher et al., 2005a). Activation of nuclear hormones via pharmacological means or genetic manipulation often upregulates drug Abcc transporter expression in liver (Faber et al., 2003; Maher et al., 2005a).

For example, microsomal enzyme inducers, such as [2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyl 126 (PCB126), and beta-naphthoflavone], 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) increased Abcc2 expression in mouse liver (Maher et al., 2005a). Certain exposures can down-regulate Abcc transporter expression in liver, such as lipopolysaccharide (LPS) administration. Studies mentioned above are adulthood exposure studies, only very few studies describe whether transporters and hepatic clearance mechanisms are regulated by early in life exposure that can contribute to epigenetic mechanisms.

Drug transporters and Phase-II enzyme expression in liver differs from neonates to adults in rodent and humans (Coughtrie et al., 1988; Cui et al., 2010). In rodents, Abcc transporter expression change with age (Maher et al., 2005b; Cheng et al., 2007). In neonates and children, Phase II enzymes such as UGTs expression is low compared to adults (Coughtrie et al., 1988). Similarly drug transporters such as Abcc family transporters, have low hepatic expression in neonates compared to adult mice (Maher et al., 2005b). In rodents, gender dependent Abc transporter expression is observed in liver.

For example, Abcc4 is highly expressed in females compared to males (Maher et al., 2005b). In rats, age dependent differences in Phase-II and drug transporter expression in neonates are taught to be responsible for cardiac glycoside induced toxicity (Guo et al., 2002; Maher et al., 2005b). Low expression of hepatic Abcc2 was taught to be one of the reasons for occurrence of neonatal jaundice (Maher et al., 2005b).

2. Obesity:

Obesity is metabolic disease characterized with increased body mass index ($BMI \geq 30$) (2010). Obesity is a hallmark disease of metabolic syndrome and widely considered as a complex condition. There is growing concerns of obesity affecting people worldwide and approximately above 30% of US population was effected by obesity of which 5% are considered as morbidly obese. During obesity, fat accumulation in liver (steatosis) was observed which represents non-alcoholic fatty liver (Wanless and Lentz, 1990). It is well established that during obesity and other metabolic disorders several genes expression changes take place particularly in liver. Few studies documented the coordinate regulation of drug transporter and nuclear receptor expression in steatosis. Altered nuclear hormone expression in fatty liver, was taught to be one of the factors responsible for change in metabolic genes (Cheng et al., 2008; More and Slitt, 2011). Obesity and other metabolic syndrome components are also characterized with altered levels of metabolism-related hormones such as insulin, resistin, glucagon and amylin levels (Azuma et al., 2003; Reinehr et al., 2007). Hyperinsulinemia was observed during obese

and diabetic conditions. No studies have shown relation between metabolism-related hormones role in regulating drug transporters. Recently, drugs that target these metabolism-related hormones are developed for treating obesity and diabetes (Schmitz et al., 2004). There is a need to identify whether these metabolism-related hormones have role in regulating gene expression changes particularly drug transporter expression during development of obesity.

3. Leptin and rodent models of obesity:

There are of several models of obesity in rodents such as diet induced obesity or genetically modified obesity. Ob/ob mice are genetically modified obese model, these mice have a spontaneous mutation in Ob gene (leptin). Leptin a hormone produced by adipose tissue both in human and rodent plays crucial role in food consumption (Frederich et al., 1995; Kennedy et al., 1997). Leptin regulates food consumption by acting on the central nervous system as a negative feedback system for body fat accumulation (Elmquist et al., 1998; Schwartz et al., 2000). Decreases in fat and body weight were observed with leptin supplements in normal and mice lacking Ob gene (Muzzin et al., 1996; Elmquist et al., 1998; Friedman and Halaas, 1998; Ahima and Flier, 2000; Schwartz et al., 2000). Leptin levels in body correspond to amounts of fat tissue. Leptin acts through membrane receptors (Ob-R), there are several subtypes of OB-R receptors they differ by length, location and functionality (Kastin et al., 1999; Hileman et al., 2000; Martin et al., 2008). Obese Zucker rat have mutated leptin receptor (fa, Ob-R gene), these rats display similar

conditions as ob/ob mice. Both in rodent and humans, there is an increase in circulating leptin levels proportional to increase in adiposity in diet induced obesity (Li *et al.*, 1997) . Ob-Re is a soluble leptin receptor isoform and several other Serum Leptin Interacting Proteins (SLIPs) play crucial roles in regulating leptin activity both centrally and peripherally (Lammert *et al.*, 2001; Zastrow *et al.*, 2003; Chen *et al.*, 2006). Leptin has no central action in neonatal mice despite fact that there are high levels of circulating levels of leptin. In neonates, high expression of OB-Re leptin receptor, which act as leptin inhibitory protein was thought to be responsible for lack of leptin action over CNS during that age (Ahima *et al.*, 1998; Pan *et al.*, 2008). These studies suggest that leptin has no or minimal regulation on food consumption in neonates, which is contrary to leptin action in adults.

Adult ob/ob mice have well characterized models for obesity diabetes and steatosis that is part of non-alcoholic fatty liver disease. Ob/ob mice have characteristic features such as hyperphagia, hyperglycemia, glucose intolerance, elevated plasma insulin, subfertility, hypometabolism and hypothermia (Lindstrom, 2007). All these physiological changes were predominantly observed in ob/ob mice from four weeks of age (Dubuc, 1976). Previous studies show that leptin has minimal or no central action until mice are two weeks old; moreover ob/ob mice did not show any significant physiological changes until they reach adolescence. This suggests neonatal Ob mice should have normal physiological and gene expression changes as normal mice. Drug transporter expression is known to alter during several

metabolic conditions. Both genetically modified and diet-induced obesity alters drug transporter expression. Analyzing ontogeny of Ob mice hepatic drug transporter expression will provide an insight how lack of leptin action and increased levels of serum hormone levels can affect drug transporters expression.

4. Bisphenol A:

Bisphenol A (BPA) is a monomer used in plastic manufacturing. According to National Health and Nutrition Examination Survey, 92.6% of 2500 participants have BPA in urine samples (Calafat *et al.*, 2008). BPA exposure during gestation and lactation periods resulted in dysregulation of several aspects in pups. The negative effects of BPA exposure are considered to be higher in children compared to adults may be due to low expression of elimination pathway. Adverse effects of BPA exposure includes increased body weights in females, early onset of puberty, alterations in mammary glands and reproductive glands, changes metabolic features, causes insulin resistance, increases adipogenesis and predisposes to metabolic syndrome in pups (Alonso-Magdalena *et al.*, 2006; Somme *et al.*, 2009; Wei *et al.*, 2011). All these adverse effects shown by BPA exposure involve multiple mechanisms, such as epigenetic modulation and endocrine disruption. In mice, methyl donor supplementation along with BPA treatment has muted epigenetic modulation shown by BPA (Dolinoy *et al.*, 2007). BPA acts as an endocrine disruptor by mimicking estrogen and binds to estrogen receptors (ER) such as ER-alpha and beta. BPA is also known to act as an antagonist for thyroid

hormone receptors. BPA developmental exposure in A^{vy} mice has resulted in change in coat color of pups through epigenetic modulation which was muted by methyl donor supplementation along with BPA exposure (Dolinoy et al., 2007). BPA developmental exposure in these A^{vy} mice altered sexual dimorphic gene expression and sexual traits expressed in adult mice (Jasarevic et al.; Mao et al.).

BPA is highly metabolized in gut and liver, and eliminated by urinary and fecal excretion (Vandenberg *et al.*, 2009). Drug transporters play key role in elimination of BPA from body, as they are involved in elimination of BPA metabolites. BPA-glucuronide and BPA-sulfate are the major metabolites formed in humans and mice (Vandenberg et al., 2009). The Ugt2b family and Sult1a1 enzymes are major biotransformation enzymes involved in BPA conjugation (Nishiyama et al., 2002; Vandenberg et al., 2009). Early developmental toxicity caused by BPA in neonates may be due to low expression of several Phase II enzymes compared to adults (Hines, 2008). Decrease in these Phase-II and drug transporter expression in liver may cause accumulation of BPA in body. BPA metabolite elimination differs from rodent to human and non-human primates. In rodents, BPA primarily is excreted by the biliary route whereas in human BPA undergoes urinary excretion (Vandenberg et al., 2009). Some *in vitro* studies performed in hepatic carcinoma cells such as HepG2 cells showed BPA exposure has altered ABC drug transporter expression (Hanet *et al.*, 2008). To date, there are no studies reported or performed showing effects of BPA exposure on hepatic drug

transporters, yet transporters are vital to maintaining systemic levels of circulating hormones. Disruption of hepatic transporter expression may be one of the mechanisms by which endocrine disrupting compounds, such as BPA, elicit hormone-disrupting effects. Evaluating whether BPA developmental exposure modulates hepatic drug transporter expression or not will provide valuable information about how environmental toxicant exposure through maternal diet affects pups xenobiotic and hormone metabolism.

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MANUSCRIPT-I

Characterizing Liver Transporter Expression in Metabolic Syndrome: Correlations between phenotype, serum hormones, and transcription factor expression

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ABSTRACT

Purpose: Obesity is a predominant risk factor for metabolic syndrome, which is defined as a cluster of risk factors that occur simultaneously and increase the likelihood of coronary artery disease, stroke, and type-2 diabetes. One manifestation of metabolic syndrome is the development of hepatic steatosis in conjunction with insulin resistance. In recent years, the occurrence of obesity in the adult population has significantly increased, which warrants the need for better drug efficacy and toxicity prediction. The purpose of this study was to determine whether clinical biomarkers and hormones could correlate with nuclear receptor and transporter-related pathways at different ages, in order to better predict altered drug metabolism or disposition.

Methods: Livers from male and female C57BL/6 and ob/ob mice littermates at 1, 3, 4 and 8 weeks of age were collected. Serum hormone and mRNA levels were analyzed using a Luminex platform. Correlation between physiological changes and gene expression was performed using canonical correlations.

Results: Significant ontogenic changes in both C57BL/6 and ob/ob mice were observed during and post-weaning. In males and females, the ontogenic pattern started to differ from week 3, but significant changes were observed at week 4 and 8 in ob/ob mice compared C57BL/6 mice. Significant correlations were observed between physiological changes, hormones, and gene expression changes during development of obesity.

Conclusion: The correlation between physiological changes and gene expression indicate metabolism-related hormones can regulate or be co-regulated with genes involved in drug metabolism and transport. Specifically, in males correlations indicate serum resistin and glucagon can regulate hepatic Abc transporter expression.

INTRODUCTION

Obesity is a metabolic disease characterized by an increased body mass index ($BMI \geq 30$). Obesity is a predominant risk factor for metabolic syndrome (MetS), which encompasses increase in body weight, adipose tissue mass, insulin resistance, and serum hormone levels (Grundy, 2004). One of the manifestations of metabolic syndrome is the development of hepatic lipid accumulation (e.g. steatosis), which represents non-alcoholic fatty liver disease (NAFLD) in conjunction with insulin resistance (Wanless and Lentz, 1990). There is a growing concern about absorption, distribution, metabolism, and excretion (ADME) in the obese population, as studies reveal an altered ADME in people affected with obesity and metabolic diseases (Brill et al., 2012). *Ob/ob* mice have a mutation in the *Ob* gene that encodes for the leptin, resulting in a phenotype that has many characteristics common to MetS. Due to lack of leptin, *ob/ob* mice exhibit hyperphagia, profound weight gain, hyperglycemia, glucose intolerance, elevated plasma insulin and severe hepatic steatosis (Lindstrom, 2007). Most of these changes are observed predominantly in *ob/ob* mice from four weeks of age (Dubuc, 1976).

Drug transporters are membrane bound proteins that facilitate both uptake and efflux of xenobiotics, endogenous compounds and their metabolites in various tissues including liver. Hepatic transporter expression is an important determinant in maintaining systemic balance of endogenous compounds such as bile acids, hormones, and bilirubin (Lecureux et al., 2009). ATP-binding cassette (Abc) transporters are kind of drug transporters, which are ATP-

dependent membrane proteins that transport a wide variety of compounds. Multiple hepatic conditions can alter drug transporter expression, such as obesity, oxidative stress, inflammation, drug-induced liver injury, and environmental toxicants (Geier et al., 2003; Aleksunes et al., 2008; Cheng et al., 2008). Previous studies document alterations in drug transporter and drug metabolizing enzyme expression (DME's) in obese and diabetic conditions (Cheng et al., 2008; More and Slitt, 2011).

In liver, transcription factors, such as pregnane X receptor (Pxr, Nr1i2), constitutive androstane receptor (Car, Nr 1i3), farnesoid X receptor (Fxr, Nr1h4) and nuclear factor E2-related factor 2 (Nrf2, Nfe2l2) regulate the basal and inducible expression of biotransformation enzymes and Abc transporters (Klaassen and Slitt, 2005). For example, Pxr and Car upregulate Cyp3a11 and Cyp2b10 expression, whereas Nrf2 upregulates Nqo1 gene transcription and expression (Aleksunes et al., 2006). With regard to transporters, hepatic Abcc2-4 induction by microsomal enzyme inducers is observed to be Nrf2-dependent (Maher et al., 2005a). Prototypical Pxr activators upregulate hepatic Abcc2, 3, Slc10a1 and Slco1a4 expression (Cheng et al., 2005b; Maher et al., 2005a; Cheng et al., 2007), whereas Car activators upregulate Abcc2-6 in liver (Cheng et al., 2005b; Maher et al., 2005a). However, the mechanisms by which transcription factors regulate transporter expression during development of fatty liver disease are largely not well described. An increase in Pxr, Car and Nrf2 mRNA expression with no changes in binding to consensus sequences was observed in livers of 9 week old ob/ob mice

compared to C57BL/6 mice (Xu et al., 2012), suggesting other mechanisms may be involved in the coordinate regulation of drug transporter and transcription factor expression in steatosis.

Obesity alters levels of metabolic hormones, such as resistin, glucagon, insulin and incretins (Starke et al., 1984; Azuma et al., 2003; Reinehr et al., 2007), which could help explain the observed changes in gene expression. Known consequence of obesity is insulin resistance accompanied by hyperglycemia (Kahn et al., 2006). Although increased incretin such as glucagon like peptide-1 (GLP-1) levels are observed in obese people, GLP-1 activity associated with insulin secretion is decreased compared to lean individuals (LaFerrere et al., 2007). Several therapies that target these hormones have been identified for treatment of obesity and other metabolic diseases (Schmitz et al., 2004). During obesity correlations have been identified between changes in serum resistin, leptin and insulin levels (Pantsulaia et al., 2007). As more incretins based therapies are being utilized to manage diabetes a component of Mets, it is critical to understand whether incretin hormones could modulate drug disposition.

Ob/ob mice are commonly used to model MetS and fatty liver disease (Lindstrom, 2007). This study aims to correlate typical clinical endpoints and metabolic hormones with hepatic transcription factors, prototypical drug metabolizing enzyme (DME), and transporter mRNA expression. The findings of this study provide potential insight into possible measures and serum

biomarkers that can be used to predict potential ADME changes in obese patients.

MATERIALS AND METHODS

Animals and husbandry. Heterozygous mice were mated and offspring were genotyped for sex and mutation of the leptin gene. Tissues from male and female wild type (C57BL/6J) and homozygous (ob/ob) were collected at age of week 1, 3, 4, and 8 (n=4-5 per group). Blood was collected and serum was obtained after centrifugation at 5,000 rpm for 5 minutes at 4°C. Livers were collected, snap frozen in liquid nitrogen, and stored at -80°C for future analysis. All animal experiments were approved by University of Rhode Island Institutional Animal Care and Use Committee (IACUC).

Hematoxylin and eosin staining. After collection, a small section of liver from the central lobe was fixed in formaldehyde for 24 h and then transferred to 75% ethanol prior to paraffin embedding. Paraffin-embedded tissues were cut to approximately 5 micron sections, and stained with hematoxylin and eosin by standard histology protocols (AML Laboratories, Rockland, MD).

RNA extraction. Total RNA from livers was isolated by phenol-chloroform extraction method using RNA-Bee reagent (Tel-Test Inc., Friendswood, TX), according to the manufacturer's protocol. RNA concentration was quantified by absorbance at 260 nm using a Nanodrop ND1000 (Thermo Fisher Scientific, Waltham, MA) and the samples were diluted to 1 µg/µL. Formaldehyde–agarose gel electrophoresis followed by UV illumination was used to visualize RNA and confirm integrity.

QuantiGene® multiplex suspension array. Mouse liver mRNA expression was determined using a QuantiGene® Plex 2.0 assay (Panomics Inc., Fremont, CA) with a Bio-Plex System Array reader with Luminex 100 xMAP technology, and data was acquired using Bio-Plex Data Manager Software (Bio-Rad, Hercules, CA). Assays were performed according to the manufacturer's protocol (Panomics, Inc.). The optimal RNA input was determined prior to running the assay. Briefly, 500 ng of total RNA was incubated overnight at 53°C with X-MAP beads containing oligonucleotide capture-probes, label extenders, and blockers. On the next day, beads and bound target RNA were washed and subsequently incubated with amplifier at 46°C for 1 hr. Next, samples were washed and incubated with the label (biotin) at 46°C for 1 hr. Samples were washed and incubated with streptavidin-conjugated R-phycoerythrin, which binds biotinylated probes, and incubated at room temperature for 30 min. Streptavidin-conjugated R-phycoerythrin fluorescence was then detected for each analyte within each sample. Data was normalized with the average week 1 expression in C57BL/6 mice and expressed as arbitrary units (AU).

Branched DNA amplification (bDNA) assay. Relative bile salt-export pump (Abcb11, Bsep) and Na⁺-taurocholate cotransporting polypeptide (Slc10a1, Ntcp) mRNA levels were quantified using bDNA assay using previously described probesets (Cheng et al., 2007). All reagents for analysis including lysis buffer, amplifier/label probe diluent and substrate solution were supplied in the QuantiGene 1.0 assay kit (Panomics, Fremont, CA). Briefly, the probe

set stocks containing capture extenders, label extenders, and blockers were diluted 1:100 in lysis buffer before use. On day one, total RNA samples (10 μ g) were added to wells containing 50 μ L of capture hybridization buffer and 50 μ L of diluted probe set. The RNA was allowed to hybridize overnight with the probe set at 53°C. On day two, subsequent hybridization steps were followed as detailed in the manufacturer's protocol, and fluorescence was measured with a GloRunner™ microplate luminometer interfaced with GloRunner DXL Software (Turner Biosystems, Sunnyvale, CA). The fluorescence for each well was reported as relative light units (RLU) per 10 μ g of total RNA (Donepudi et al., 2012). Data was normalized with the average of week 1 expression in C57BL/6 mice and expressed as arbitrary units (AU).

Serum metabolism-related hormone levels. Serum metabolism-related hormones were quantified using a Millipore 10-plex kit (MMHMAG-44K) on a Bioplex® multiple array system. A custom Millipore-plex kit containing different targets such as insulin, glucagon, resistin, glucagon like peptide-1 (GLP-1), amylin and leptin was used and analyzed according to manufacturers protocol. Fluorescence was detected on a Bioplex® multiple array reader system (BioRad, Hercules, CA). Data was collected by Bioplex® manager 5.0 software and plotted as average concentration (μ g/mL).

Correlation analysis. Correlations between the mRNA levels of genes related to drug metabolism and transcription factors were performed using either Statistica 9.1 software (Stat Soft, Inc., Tulsa, OK) or canonical correlation analysis (CCA). Briefly, the canonical correlation analysis data generated

was log transformed and distributed in 3 blocks such that block 1 contained gene expression of drug transporters and phase I enzymes, block 2 contained transcription factor expression, and block3 contained physiological data-serum hormone and, glucose levels, body and, liver weights. Cross block pairwise bivariate correlations were performed between each block and heat maps were generated. Hierarchical clustering was performed using the same data with pearson correlation. Data presented as heat maps or with r value, $p \leq 0.05$ is considered as a statistically significant correlation.

Statistical analysis. The statistical significance between groups was determined by factorial ANOVA followed by a Duncan's Multiple-range post hoc test, using Statistica 9.1 software (Stat Soft, Inc., Tulsa, OK). Data are presented as mean \pm SE, with $p \leq 0.05$ considered statistically significant.

RESULTS

Tissue and body weights, blood glucose levels: Figure 1A illustrates the body weights of male and female C57BL/6 and ob/ob mice. At week 1 of age, body weight was similar between all groups. At weeks 4 and 8, ob/ob mice had significantly higher body weight compared to C57BL/6 mice, as anticipated. Figure 1B illustrates serum glucose levels each group of mice. A 2.3-fold age-dependent increase in serum glucose levels was observed at 8 weeks compared to week 1 C57BL/6 mice. In male ob/ob mice, serum glucose levels increased with age at week 4 and 8 by 2.1 and 4.5-fold respectively, compared to week 1 ob/ob male mice. In female ob/ob mice, serum glucose levels increased with age at week-3, 4 and 8 by 1.7, 1.9 and 3.8-fold respectively compared to week 1 female ob/ob mice. Serum glucose levels in both male and female ob/ob mice were significantly increased at week 8 compared to C57BL/6 counterparts by 1.3-fold, these changes in serum glucose levels between ob/ob mice and C57BL/6 mice were not observed at early ages.

Ontogeny of serum hormones in C57BL/6 and ob/ob mice. Figure 1C illustrates serum hormone changes observed in both male and female C57BL/6 and ob/ob mice from weeks 1, 3, 4 and 8. Serum hormone levels were similar between C57BL/6 and ob/ob mice at one week of age. In male C57BL/6 mice, glucagon, resistin and GLP-1 decreased with age, but insulin and amylin did not. In male C57BL/6 mice, serum glucagon, resistin, and GLP-1 levels were decreased by ~78% from week 3 compared to week 1.

However, in male ob/ob mice, serum insulin and amylin increased with age after week 4 by 4-fold. Also, serum insulin, glucagon, resistin, GLP-1 and amylin levels were significantly elevated in male ob/ob mice compared to male C57BL/6 mice from 4 weeks of age.

In female C57BL/6 mice serum glucagon, resistin and GLP-1 levels decreased with age. However, serum insulin and amylin levels were similar at all weeks assessed. In female ob/ob mice, insulin and amylin increased after four weeks of age while glucagon, resistin, and GLP-1 levels decreased by 57%, 31% and 40% respectively, compared to 1 week old female ob/ob mice. Similar to observations in males, all serum hormone levels were increased at 8 week time point in females ob/ob mice compared to corresponding C57BL/6 females.

Transporter and prototypical metabolizing enzyme expression livers of C57BL/6 and ob/ob mice. Figure 2 illustrates hepatic efflux drug transporter expression observed in both male and female C57BL/6 and ob/ob mice. In C57BL/6 and ob/ob mice ontogenic changes were observed in Abc transporter expression for Abcc1, 3, 4, 5 and Abcb11 in both males and females. Similar to physiological changes, no significant changes were observed in Abc transporters expression between one-week old male and female C57BL/6 and ob/ob mice. In male ob/ob mice, Abcc3, 4 and Abcg2 mRNA expression was increased significantly compared to C57BL/6 mice by 1.8, 7 and 2.3-fold respectively. A similar increase was observed in female ob/ob mice, with Abcc3, 4 and Abcg2 mRNA expression compared to female C57BL/6 mice. In

contrast to males, female ob/ob mice have significant decrease in Abcb11 mRNA expression by 49% compared to female C57BL/6 mice. Most of these significant changes in both male and female ob/ob mice were observed from 4 weeks of age.

Figure 3 illustrates hepatic uptake drug transporter expression in both male and female C57BL/6 and ob/ob mice. In both male and female C57BL/6 mice, Slco1a1 expression increased significantly at week 8 compared to week 1 by 219 and 95-fold respectively. However these changes in Slco1a1 mRNA levels were not observed in both male and female ob/ob mice. Interestingly, in 8-week old male and female ob/ob mice, Slco1a1 expression was significantly decreased by 97 and 98% respectively, compared to their C57BL/6 counterparts. In male and female, C57BL/6 and ob/ob mice, ontogenic changes in Slco1a4 were observed only at 3 and 4 weeks of their age but not in week 8 old mice compared to week 1.

Figure 4 illustrates hepatic DME expression observed in both male and female C57BL/6 and ob/ob mice. In male and female ob/ob mice Cyp2b20 and 4a14 mRNA expression significantly increased with age compared to week 1 whereas no ontogenic changes were observed in C57BL/6 mice. Changes in mRNA expression with age in Cyp3a11 were observed from 3 weeks of age whereas changes in Cyp2b20 and 4a14 mRNA expression were observed from 4 weeks of age. Male ob/ob mice also showed a significant increase in cyp2b20 and 4a14 expression from 4 weeks of age compared to their C57BL/6 counterparts by 4.3 and 2.2-fold respectively. Female ob/ob mice

had significant increase in expression of Cyp3a11 (1.3 fold) and Cyp2b20 (1.8 fold) expression compared to C57BL/6 mice at only week-4. Cyp4a14 mRNA expression in female ob/ob mice significantly greater at both 4 and 8 weeks by 2.5 and 3.1-fold respectively compared to female C57BL/6 mice.

Hepatic transcription factor expression of C57BL/6 and ob/ob mice.

Figure 5 illustrates the hepatic transcription factor mRNA expression levels in livers of male and female C57BL/6 and ob/ob mice. In male C57BL/6 and ob/ob mice, there were no significant ontogenic changes in transcription factor mRNA expression, except Nrf2 and Ppar- α . In male ob/ob mice, Nrf2 mRNA expression increased at week-8 by 2 fold compared to week-1. At week-8, Car, Fxr and Nrf2 mRNA expression significantly increased by 2-3 fold, in male ob/ob compare to male C57BL/6 mice. In female C57BL/6 mice, Pxr mRNA expression decreased with age by 51% compared to week-1, however this is not observed in female ob/ob mice. In female ob/ob mice, only Fxr mRNA levels significantly increased by 2.5 fold at 3 and 4 weeks of their age compared to week-1. In females no significant changes were observed in transcription factors expression between ob/ob and C57BL/6 mice.

Correlation analysis between gene expression changes and phenotypical changes.

Role of gender and leptin in ontogenic changes in hepatic gene expression pattern is depicted pictorially using heat maps (Figure 6 & 7). Gene expression values were log transformed to generate heat maps using R-language. These heat maps strongly indicate leptin and gender plays a huge role in the regulation of hepatic genes involved in metabolism and

disposition. Difference in ontogenic changes in ob/ob mice and C57BL/6 mice in both male and female illustrates influence of leptin over peripheral tissues apart its regulation of satiety at central nervous system. Table 1 & 2 illustrates correlations between physiological factors, such as metabolic hormone levels, serum glucose, and body and liver weights were performed using canonical correlations. Canonical correlations were performed to identify physiological factors, which may have a potential role as a biomarker during development of obesity (Table 1 & 2). In males, serum resistin and insulin levels correlate with all physiological factors, whereas in female mice, serum resistin, insulin and amylin correlate with other physiological factors during development of obesity. In both males and females, these correlations between physiological factors suggest that changes in serum resistin levels may be associated with development of obesity irrespective of gender.

Furthermore, canonical correlations were performed between physiological factors and gene expression to identify relationships between changes in gene expression during development of obesity and common parameters used to assess MetS. Figure 8 illustrates correlations for physiological factors such as hormone level, body, and liver weights with drug transporter and DME expression in male and female mice presented as heat maps. In males and females, serum amylin levels correlated with Abcc4, Abcg2, Cyp2b20 and 4a14 expression. Correlation between other physiological factors and drug transporters expression differ between males and females. For example, serum resistin levels correlated with Abcc1, 4, 5, Abcb1a, Abcb11, Slco1a1,

Cyp3a11 and 4a14 expression in male mice, whereas resistin levels correlated to Abcc1, Abcb11, Slco1a1, 1a4, Slc10a1, Cyp3a11, 2b20 and 2e1 in females. Furthermore, serum insulin concentrations correlated to Abcc4, Abcg2, Cyp2b20 and 4a14 expression in males whereas it is correlated to Abcc4, Abcg2 and Slco1a1 expression in females. Serum glucagon concentrations correlated with Abcc1, 4, Abcg2, Abcb1a, Abcb11, Slco1a1, Cyp3a11 and Cyp4a14 expression in males, but correlated to Abcc6, Abcb11, Slco1a4, 1b2 and Slc10a1 expression in females. In males, serum GLP-1 concentrations were correlated with only Abcb11, Slco1a4, Cyp3a11 and 2e1 expression whereas in females it is correlated with Abcc2, 3, Abcb11, Slco1a1, 1a4, 1b2, Cyp3a11, 2b20 and 2e1 expression. In males, serum glucose concentrations correlated to Abcc1, 3, 5, 6, Abcb1a, Abcb11, Cyp3a11 and 2b20 expression whereas it is correlated only to Abcc3-5, Cyp3a11 and 2b20 in females. Both body and liver weights correlated to Abcc1, 3, 5, 6, Abcb1a, abcb11, Slco1a1, Cyp3a11 and 2b20 expression in male mice, whereas in females they correlated with Abcc1, 3- 6, Cyp3a11 and 2b20 expression.

Figure 9 illustrates the correlation (heat maps) between transcription factors with drug transporters and phase I enzymes expression in male and female mice. Correlation between transcription factors, drug transporters, and DME expression was performed using canonical correlations and the data generated is presented as a heat map. In males, during development of obesity and MetS, expression patterns of all Abc transporters except Abcb11 were correlated with all transcription factors analyzed with minor exceptions.

In male mice, Abcc3 expression did not correlate with Pxr, Car, Ppar- α , and Ppargc1 α expression, whereas Abcc5 is not correlated with Fxr. Correlation between Abc transporters and transcription factors showed gender dependent changes during development of obesity. Interestingly, in female mice only Abcc2, 6 and Abcb1a expression was correlated with all transcription factors analyzed, whereas Abcg2 expression was correlated with all transcription factors except Car during development of obesity. In female mice, Abcc3 expression was correlated to Car, Lxr, Fxr and Nrf2 expression, whereas Abcc4 expression was correlated to Pxr, Lxr, Fxr and Nrf2 expression. In both male and female mice, uptake transporter Slco1b2 expression was correlated to all transcription factors expression whereas Slco1a4 expression was correlated with Car, Lxr, Fxr, Nrf2 and Ppargc1 α expression. Drug metabolizing enzymes such as Cyp2e1 and 4a14 expression was correlated with all transcription factors expression in both male and female mice. In male mice, Cyp2b20 expression was correlated to Lxr, Fxr and Nrf2 expression whereas in females Cyp3a11 and 2b20 were correlated to Car, Lxr, Fxr and Nrf2 expression.

DISCUSSION

Obesity and the subsequent MetS are major concerns in the United States (Grundy, 2004). Non-alcoholic fatty liver disease (NAFLD) is a manifestation of MetS. About 15-39% of the US population is affected with NAFLD, and about 95% of the morbidly obese are diagnosed with NAFLD (Younossi et al., 2002; Collantes et al., 2004). Ob/ob mice are used to model MetS and

NAFLD because they have multiple markers that are elevated in a manner similar to humans with uncontrolled MetS – morbid obesity, markedly elevated glucose levels, insulin resistance and dysregulation of metabolic hormones, dyslipidemia, increased markers of inflammation, and hepatic steatosis (Lindstrom, 2007). Studies have shown alteration in drug transporter and drug metabolizing enzyme expression during NAFLD, which resulted in altered drug elimination (Barshop et al.; Lickteig et al., 2007; Cheng et al., 2008; Hardwick et al., 2012). Moreover, changes in hepatic uptake and efflux transporter expression in ob/ob mice is somewhat similar to changes observed in diet-induced obese mice and human steatotic livers (Cheng et al., 2008; More and Slitt, 2011).

Previous studies have shown that expression of several DMEs and drug transporters in C57BL/6 mice change with age (Cheng et al., 2005a; Maher et al., 2005b; Cui et al., 2010). In this study we characterized ontogeny of transporters along with phase-I enzymes in coordination with transcription factors in both C57BL/6 and ob/ob mice. We selected four different ages (e.g. week-1, 3, 4 and 8) to capture different times during the progression of MetS. Ontogeny of drug transporters in both male and female C57BL/6 mice we observed are similar to published studies (Cheng et al., 2005a; Maher et al., 2005b). Changes observed in transporters, phase-I enzymes, and transcription factors expression in ob/ob mice compared to C57BL/6 mice were similar to published studies (Cheng et al., 2008; Xu et al., 2012).

As mentioned earlier ob/ob mice have characteristic physiological changes. At

week-1 body and liver weights do not show any significant difference from their lean counterparts. However, increased body and liver weight were observed with development of obesity in ob/ob mice irrespective of gender (Figure 1A and 11). These observations with body and liver weights are consistent with previously published studies (Dubuc, 1976). In ob/ob mice, similar to body and liver weight changes, hyperglycemia developed with development of obesity. Liver histology was similar at week-1, steatosis was observed at week-3, but prominent changes in histology presented at week-4 (Figure 12). Metabolic-related hormones concentrations altered with development of obesity although their concentrations are similar to lean littermates at early ages. Interestingly, correlations between physiological changes showed serum resistin and insulin levels have significant correlations in both males and female mice during development of obesity. Resistin is circulating protein secreted by adipose tissue, which plays an important role in causing insulin resistance (Steppan and Lazar, 2002). In diet-induced obese mice, administration of anti-resistin antibody corrected hyperglycemia and insulin resistance, which are key factors for causing obesity (Steppan and Lazar, 2002). Our results from correlation analysis also indicates changes in serum resistin levels can be a possible biomarker, which can predict changes liver gene expression associated with MetS.

Incretins, like amylin and GLP-1, play a key role in insulin secretion and maintaining blood glucose levels. In both male and female ob/ob mice, GLP-1 levels were changed at week-8, whereas amylin levels increased from week-4.

Amylin is secreted along with insulin from pancreas and involved in maintaining serum glucose levels (Moreno et al., 2011). Changes in amylin and insulin levels in our study were observed at same time. Moreover, changes in amylin and insulin levels along with resistin showed significant correlation with other physiological parameters analyzed in females during development of obesity. As mentioned by Reinehr et al 2007, hyperamylinemia was thought to be one of the important factors in causing several metabolic abnormalities during obesity and MetS (Reinehr et al., 2007). Recently new incretin based drug therapies for MetS such as amylin and GLP-1 agonists are gaining importance (Schmitz et al., 2004). Correlation observed in our study indicates alteration in incretin levels may alter several physiological factors and hepatic metabolism-related gene expressions eliciting need to consider possible changes in xenobiotic metabolism and disposition in people undergoing multiple medications along with incretin based therapies. Post market studies Indicates, that patient under GLP-1 agonist therapies has 1.93 fold increase in occurrence of biliary infections such as cholelithiasis, which can be caused due to altered hepatic Abc transporter expression. Correlation observed in our study and post market analysis of incretin based therapies support that possibility of hepatic drug transporter regulation by metabolic hormones.

Abc transporters comprise the majority of hepatic efflux pumps, which efflux compounds from hepatocytes into bile or blood (Faber et al., 2003). In adult

mice livers, relative Abcc1 and 4 are low, whereas Abcc3 expression is moderate, and Abcc6 is high (Maher et al., 2005b). In both C57BL/6 and ob/ob mice, Abcc1 and 5 are expressed highly at week1 and decreased with age. This pattern of high expression at initial stages and decrease at later age period in expression of Abcc1 is also observed in liver regeneration after undergoing 90% hepatectomy, indicating Abcc1 to have a lesser role in adult liver (Kimura et al., 2012). Abcc3 is highly inducible basolateral efflux transporter that can cause altered vectorial disposition of xenobiotics (Slitt et al., 2003). Abcc3 and 4 play key role in efflux of several xenobiotics and endogenous compounds such as estrogen and bile acid conjugates. Abcc 4 protects hepatocyte from bile acid toxicity during cholestatic conditions (Mennone et al., 2006). In both male and female C57BL/6 mice there is a slight increase although not statistical difference was observed in Abcc3 and 4 expressions with age. In both males and females obesity increased Abcc3 and 4 mRNA levels with age as well as compared to their lean littermates.

Apical efflux transporters such as Abcc2, Abcg2, Abcb1a and Abcb11 plays major role in excretion of xenobiotics and endogenous substances from liver to bile. In both male and female mice, Abcc2 and Abcb1a mRNA levels are unaltered with age and obesity. Previous studies indicated no change in mRNA levels and increase in protein levels of Abcc2 in ob/ob mice, which is consistent with our results (Cheng et al., 2008). Abcc2 expression during obesity is species specific; in obese zucker rats Abcc2 expression decreases whereas it increases in ob/ob mice, but in humans there is no change with

obesity (Pizarro et al., 2004; Cheng et al., 2008; More and Slitt, 2011). In male ob/ob mice Abcb11 expression did not alter compared to their lean littermates whereas in females Abcb11 expression decreased with progression of obesity. Gender specific difference in Abcb11 expression pattern may be due to gender specific difference in growth hormone responsiveness in obesity (Cocchi et al., 1993) which is known to regulate Abcb11 expression (Cheng et al., 2007). Previous studies showed that Abc transporter expression is regulated by several transcription factors (Maher et al., 2005a). Interestingly, significant correlations were observed between hepatic Abc transporters and transcription factors expression during development of obesity. These correlations during development of obesity between transcription factors and hepatic efflux transporters showed gender dependent changes. Gender dependent changes in correlation between Abc transporters and transcription factors are much more pronounced in week-8 mice (Table 3 and 4).

Hepatic uptake transporter Slco1a1 mRNA levels increased with age in C57BL/6 mice whereas in ob/ob mice it remained unchanged. In both male and female ob/ob mice Slco1a1 mRNA levels decreased significantly at week-8. Previous studies shown Slco1a1 mRNA expression is androgen dependent and negatively regulated by microsomal enzyme inducers that activate transcription factors Pxr, Car, Ppar- α and Nrf2 (Lu et al., 1996; Cheng et al., 2005b). Ob/ob mice have decreased androgen levels (Swerdlhoff et al., 1976) and increase in transcription factors expression (Xu et al., 2012), which explain decrease in Slco1a1 expression compared to C57BL/6 counterparts as

they become old. Moreover in males, at week-8 age Slco1a1 expression was negatively correlated with all transcription factors that are analyzed, although these correlations are not significant (Table 3). Obesity did not affect the ontogeny of Slco1a4, 1b2 and Slc10a1, which is contrary to previously published studies. Differences in our results are may be due to age, previously published studies showed altered hepatic Slco1a4, 1b2 and Slc10a1 expression in week-11 old mice (Cheng et al., 2008), moreover hepatic uptake transporters are shown to change their expression pattern with age (Fu et al., 2012).

Several studies have shown correlations and alteration in serum hormone levels during metabolic disorders. Studies also showed hormones such as estrogen, progesterone and androgens could alter hepatic uptake and efflux transporter expression (Geier et al., 2003; Kalabis et al., 2007). No studies have documented correlation with change in drug transporter expression with metabolism-related hormones, which are known to change in obesity. Acute or chronic administration of metabolism-related hormones such as glucagon and GLP-2 altered transporter expression and function. GLP-2 an incretin secreted along with GLP-1, which acts majorly on intestines was shown to regulate Abcc2 expression in intestine (Villanueva et al., 2010). In human and rat hepatocytes, short-term glucagon treatment altered Abcc3 function (Chandra et al., 2005). Not only transporters but also phase-II enzymes such as glutathione transferases expression were altered with glucagon and insulin treatment (Kim et al., 2003), indicating metabolism related hormones have a

potential to alter drug metabolism and related gene expression. In males, serum resistin, glucagon and glucose levels are correlated with most of hepatic Abc transporter expression compared to serum amylin, insulin and GLP-1 levels. In females, serum glucose showed more correlations with hepatic Abc transporter expression than serum metabolism-related hormones. Apart from serum metabolism related hormones and glucose levels, body and liver weights have shown high correlation with all hepatic Abc transporters analyzed (Figure 8). Not only hepatic Abc transporters but overall, serum glucose, and body and liver weight stood as a markers for change in gene expression and physiological factors during development of obesity (Figure 10). In females, during development of obesity hepatic uptake transporter mRNA expressions were correlated with serum levels of resistin, glucagon and GLP-1 (Figure 8B). These correlations indicate that not only sex related hormones such as estrogen and testosterone (Lu et al., 1996; Geier et al., 2003) but also hormone involved in energy metabolism such as resistin, glucagon and GLP-1 have a role in regulation hepatic uptake transporters.

Phase-I DMEs such as Cyp3a11 and 2e1 were not altered with obesity. Cyp2e1 expression during obesity and diabetes is species-specific. In humans, Cyp2e1 is increased during obesity and diabetes whereas in mice it either remained unchanged or decreased (Enriquez et al., 1999; Wang et al., 2003; Cheng et al., 2008). In both male and female ob/ob mice Cyp4a14 expression increased with development of obesity. Cyp4a plays a key role in fatty acid metabolism and is shown to be upregulated in adult male ob/ob mice

(Cheng et al., 2008). In males, Cyp2b20 mRNA levels increased with development of obesity compared to C57BL/6 mice whereas in female Cyp2b20 mRNA levels remained unchanged. These gender specific changes in Cyp2b20 are observed in other model such as treatment with phenobarbital, which is an inducer of Cyp2b family (Larsen et al., 1994; Cheng et al., 2008). Insulin treatment altered Cyp3a, 2e1 and 4a expression in hepatocytes (Kim et al., 2003), indicating metabolism-related hormones can regulate phase-I enzyme expression. Our results indicated that in female mice Cyp enzyme expression during development of obesity is mostly correlated to resistin and GLP-1, whereas in males these correlations are variable. Sexually dimorphic regulation of Cyp enzymes might be responsible for gender specific differences in expression and correlations with metabolism related hormones (Hernandez et al., 2009).

In summary, ob/ob mice are indistinguishable from their lean littermates at week-1. Interestingly, during week1 in both male and females there are no significant changes in transporter, DME's and transcription factors mRNA levels. Not only mRNA levels, even physiological changes such as steatosis, metabolism-related hormone levels, body and liver weight are similar between ob/ob and C57BL/6 mice at week-1. In both males and females, although ontogeny pattern started to differ from week-3 in ob/ob mice compared C57BL/6 mice, significant changes were observed in week-4 and 8. Consistent with previous studies, significant changes in physiological factors like hyperglycemia and insulin resistance were observed after weaning

(Dubuc, 1976; Lindstrom, 2007). Correlation in drug transporters and DME's expression with metabolism-related hormones in a model of obesity and MetS indicates changes in these hormones can alter ADME of xenobiotics and endogenous compounds. Although physiological changes analyzed did not show up as a biomarker for prediction in gene expression changes, the correlation indicated that metabolism-related hormones analyzed were either co-regulated or involved in regulation of hepatic gene expression, which needs further investigation. Finally, this study gives an idea about how changes in expression of drug metabolism related genes could occur at different stages of obesity and diabetes development.

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Figure 1. Effect of age and leptin deficiency on phenotypical changes in C57BL/6 and ob/ob mice. (A) Body weights of both male and female, C57BL/6 and ob/ob mice at week1, 3, 4 and 8. (B) Serum glucose levels of both male and female, C57BL/6 and ob/ob mice at week1, 3, 4 and 8. Serum glucose concentration was determined by glucose assay kit. Data is presented as average concentration (mg/dl) \pm S.E.M. (n=4-5 mice per group). (C) Metabolism-related hormone levels of both male and female, C57BL/6 and ob/ob mice at week1, 3, 4 and 8. Metabolism-related hormone levels in C57BL/6 and ob/ob mice were quantified in serum collected from female and male C57BL/6 and ob/ob mice at 1, 3, 4, and 8 weeks of age (n= 4-5 for each group). Hormones were quantified using a luminex-based assay. Data is presented as average concentration (μ g/ml) \pm S.E.M. (n=4-5 mice per group). Asterisks (*) represent a statistical difference between wild type and ob/ob of same age group and pound (#) represent a statistical difference with respect to week-1 mice ($p \leq 0.05$).

Figure 2. Effect of age and leptin deficiency on hepatic Abc drug transporters expression in both C57BL/6 and ob/ob mice. Total RNA was isolated from livers of wild-type and ob/ob mice. Hepatic ATP-binding cassette (Abc) transporter mRNA levels was quantified by the QGP 2.0 Plex assay. Data is presented as mean arbitrary units (AU) \pm S.E.M. (n=4-5 mice per group). Data presented as mean arbitrary units (AU) \pm S.E.M. (n=4-5 mice per group). Asterisks (*) represent a statistical difference between wild type and

ob/ob of same age group and pound (#) represent a statistical difference with respect to week-1 mice ($p \leq 0.05$).

Figure 3. Effect of age and leptin deficiency on hepatic uptake drug transporters expression in both C57BL/6 and ob/ob mice. Total RNA was isolated from livers of wild-type and ob/ob mice. Hepatic uptake transporter such as organic anion transport polypeptides (Oatps, Slco's) and Na⁺-taurocholate cotransporting polypeptide (Slc10a1, Ntcp) mRNA levels was quantified by the QGP 2.0 Plex assay. Data is presented as mean arbitrary units (AU) \pm S.E.M. (n=4-5 mice per group. Asterisks (*) represent a statistical difference between wild type and ob/ob of same age group and pound (#) represent a statistical difference with respect to week-1 mice ($p \leq 0.05$).

Figure 4. Effect of age and leptin deficiency on hepatic drug metabolizing enzymes (DME) expression in both C57BL/6 and ob/ob mice. Total RNA was isolated from livers of wild-type and ob/ob mice. Hepatic DME's such as Cyp3a11, 2e1, 2b20 and 4a14 expression was quantified with QGP 2.0 plex assay. Data presented as mean arbitrary units (AU) \pm S.E.M. (n=4-5 mice per group). Asterisks (*) represent a statistical difference between wild type and ob/ob of same age group and pound (#) represent a statistical difference with respect to week-1 mice ($p \leq 0.05$).

Figure 5. Effect of age and leptin deficiency on hepatic transcription factors expression in both C57BL/6 and ob/ob mice. Total RNA was isolated from wild type and ob/ob mice livers, mRNA expression of Pxr, Car,

Lxr, Fxr, Nrf2, Ppar- α and Ppargc1 α was analyzed with QGP 2.0 plex assay. Data presented as mean arbitrary units (AU) \pm S.E.M. (n=4-5 mice per group). Asterisks (*) represent a statistical difference between wild type and ob/ob of same age group and pound (#) represent a statistical difference with respect to week-1 mice ($p \leq 0.05$).

Figure 6. Heat maps for gene expression analysis performed in both male C57BL/6 and ob/ob mice. Hepatic gene expression values from week-1, 3, 4, and 8, C57BL/6 and ob/ob mice are log transformed and heat maps were generated using g-plots in R language.

Figure 7. Heat maps for gene expression analysis performed in both female C57BL/6 and ob/ob mice. Hepatic gene expression values from week-1, 3, 4, and 8, C57BL/6 and ob/ob mice are log transformed and heat maps were generated using g-plots in R language.

Figure 8. Correlation (heatmaps) between physiological factors with hepatic drug transporters and DME's expression during development of obesity in male (A) and female (B) mice. Hepatic gene expression from week-1, 3, 4, and 8, C57BL/6 and ob/ob mice are log transformed and data set was divided into two blocks. Block-1 contains hepatic drug transporters and DME's expression and block-2 contains hepatic transcription factors expression. Correlations between block-1 and 2 were performed using canonical correlations. Data displayed as heat maps with highest positive

correlations considered as black and highest negative correlation are considered as red

Figure 9. Correlation (heatmaps) between transcription factors with drug transporters and DME's expression during development of obesity in male (A) and female (B) mice. Hepatic gene expression and physiological factors such as body, and liver weights, serum metabolism-related factors such as insulin, glucagon, resistin and amylin levels from week-1, 3, 4, and 8, C57BL/6 and ob/ob mice are log transformed. Log transformed data set was divided into two block such that one of the block contains hepatic gene expression drug transporters and DME's expression and other block contains physiological factors. Correlations between both block were performed using canonical correlations. Data displayed as heat maps with highest positive correlations considered as black and highest negative correlation are considered as red

Figure 10. Hierarchical clustering of log transformed variables using Pearson correlation as a similarity measure. Gene expression of drug transporters, DMEs and physiological factors was correlated with each other. Hierarchical clustering was performed using canonical correlations.

Figure 11. Effect of age and leptin deficiency on liver weights in C57Bl/6 and ob/ob mice. Liver weight of both male and female, C57Bl/6 and ob/ob mice at week1, 3, 4 and 8. Asterisks (*) represent a statistical difference

between wild type and ob/ob of same age group and pound (#) represent a statistical difference with respect to week-1 mice.

Figure 12. Effect of age and leptin deficiency on liver pathology. A) Representative liver pathology in male C57Bl/6 and ob/ob mice at week 1, 3, 4, and 8 (n=1 for each group). B) Representative liver pathology in female C57Bl/6 and ob/ob mice at week 1, 3, 4, and 8 (n=1 for each group). Representative photomicrographs of Hematoxylin and Eosin stains of liver sections (200x).

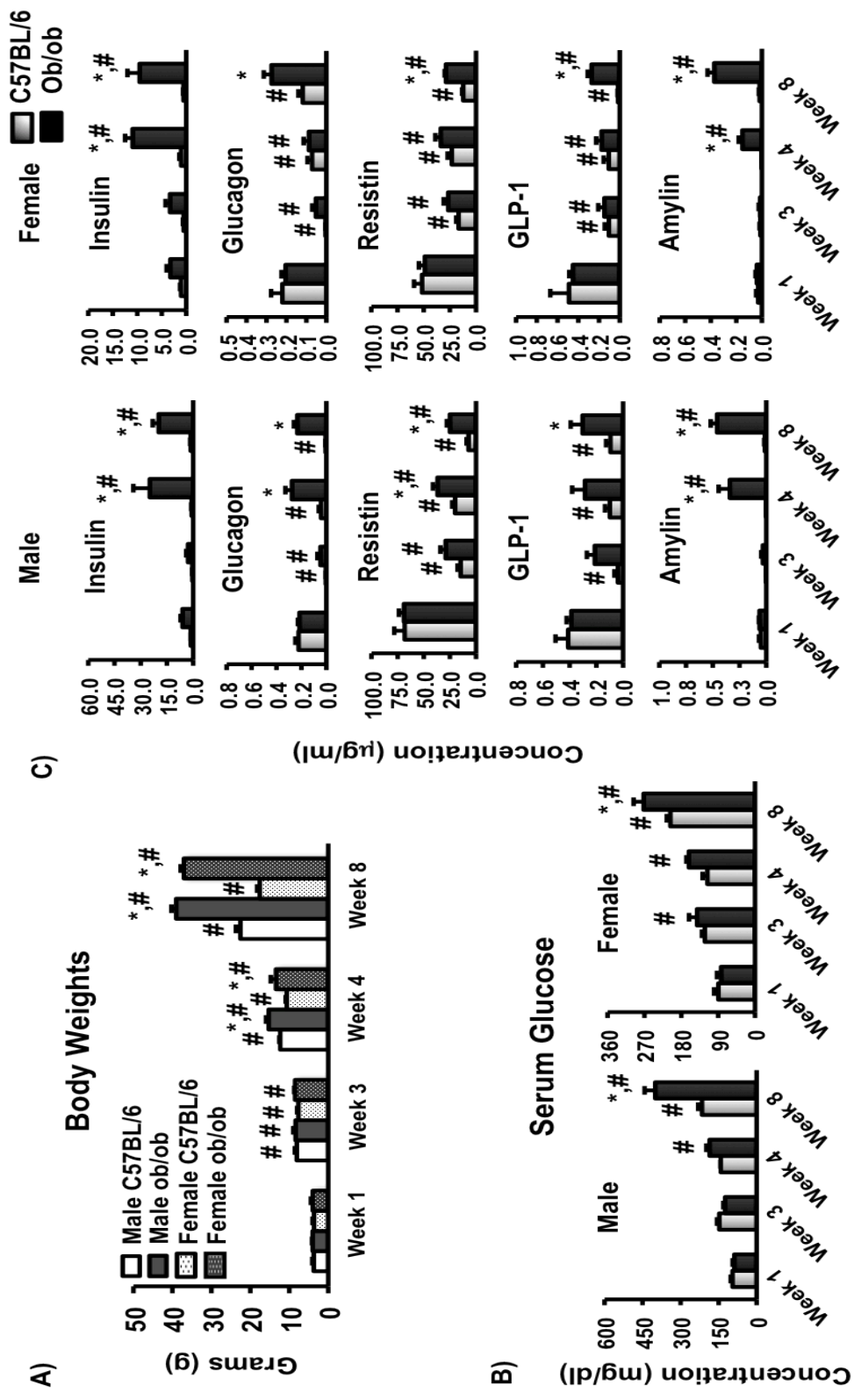


Figure 1. Effect of age and leptin deficiency on phenotypical changes in C57BL/6 and ob/ob mice

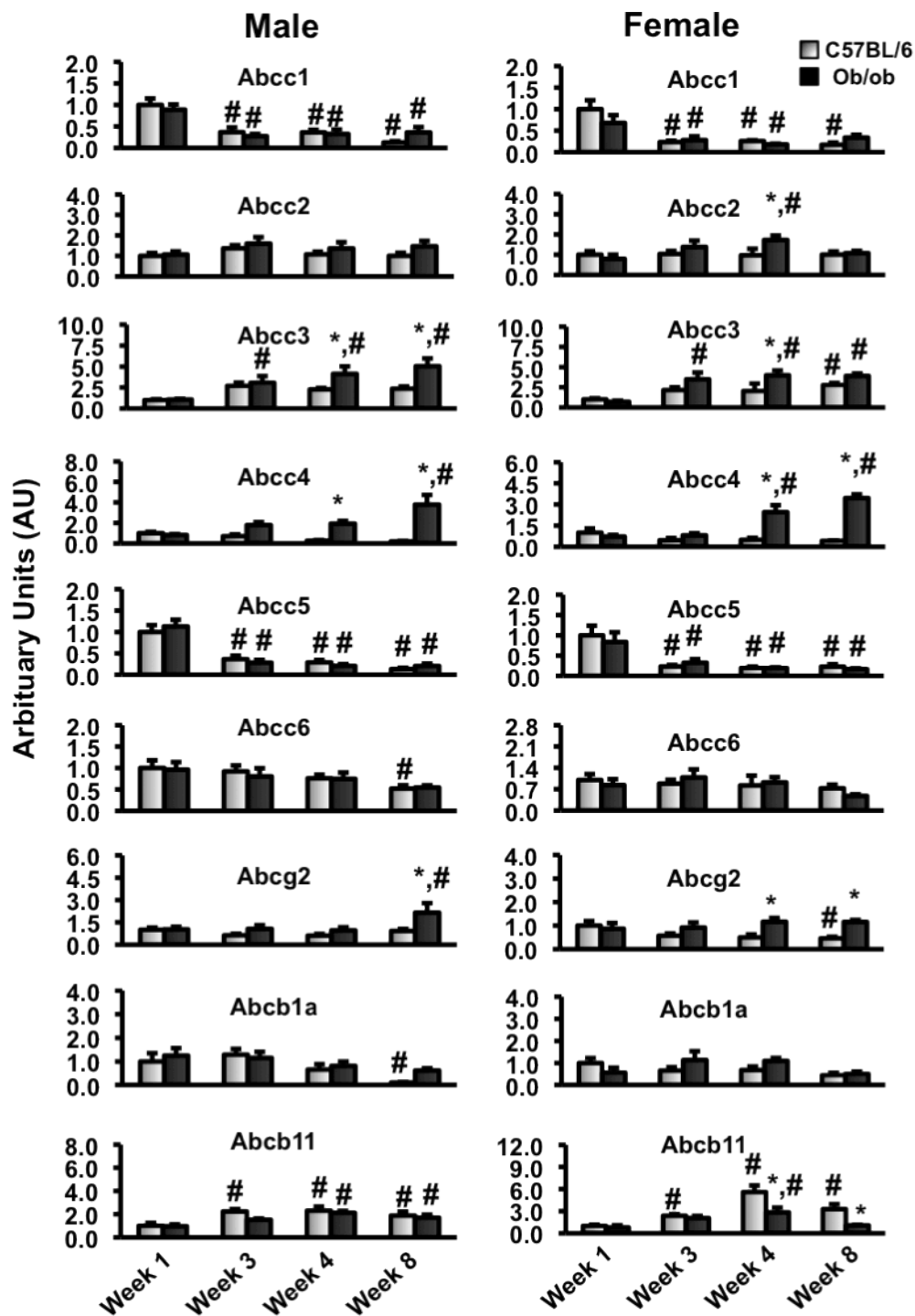


Figure 2. Effect of age and leptin deficiency on hepatic Abc drug transporters expression in both C57BL/6 and ob/ob mice

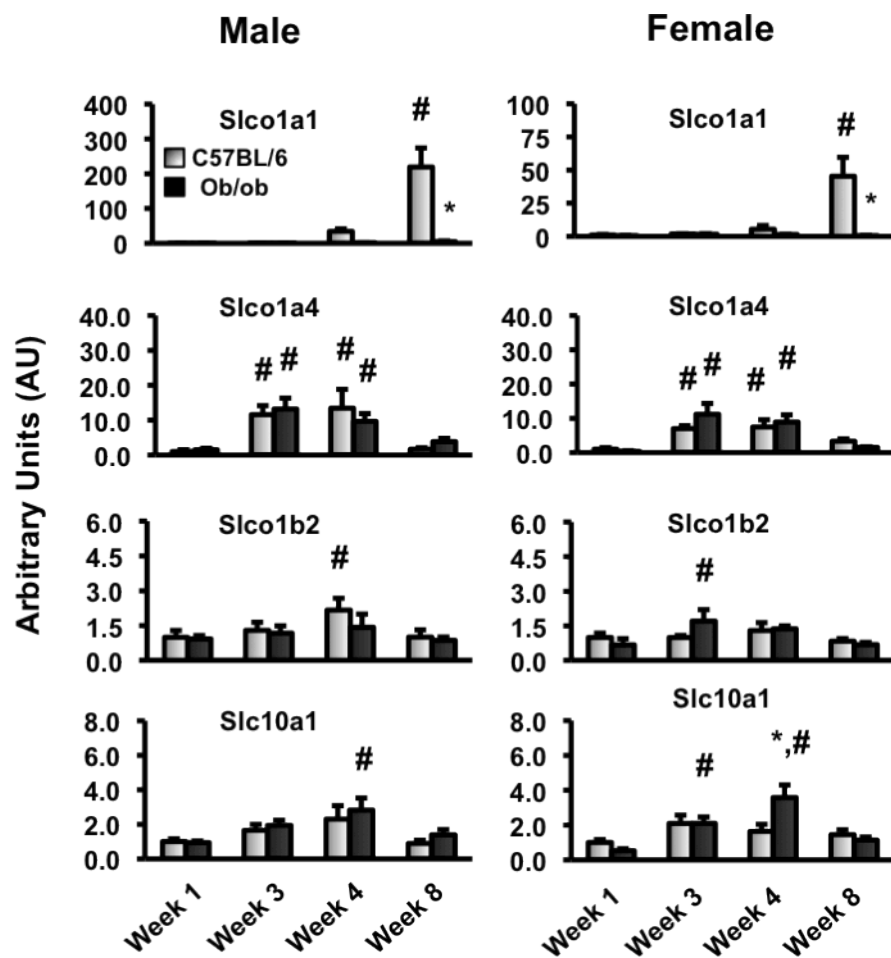


Figure 3. Effect of age and leptin deficiency on hepatic uptake drug transporters expression in both C57BL/6 and ob/ob mice

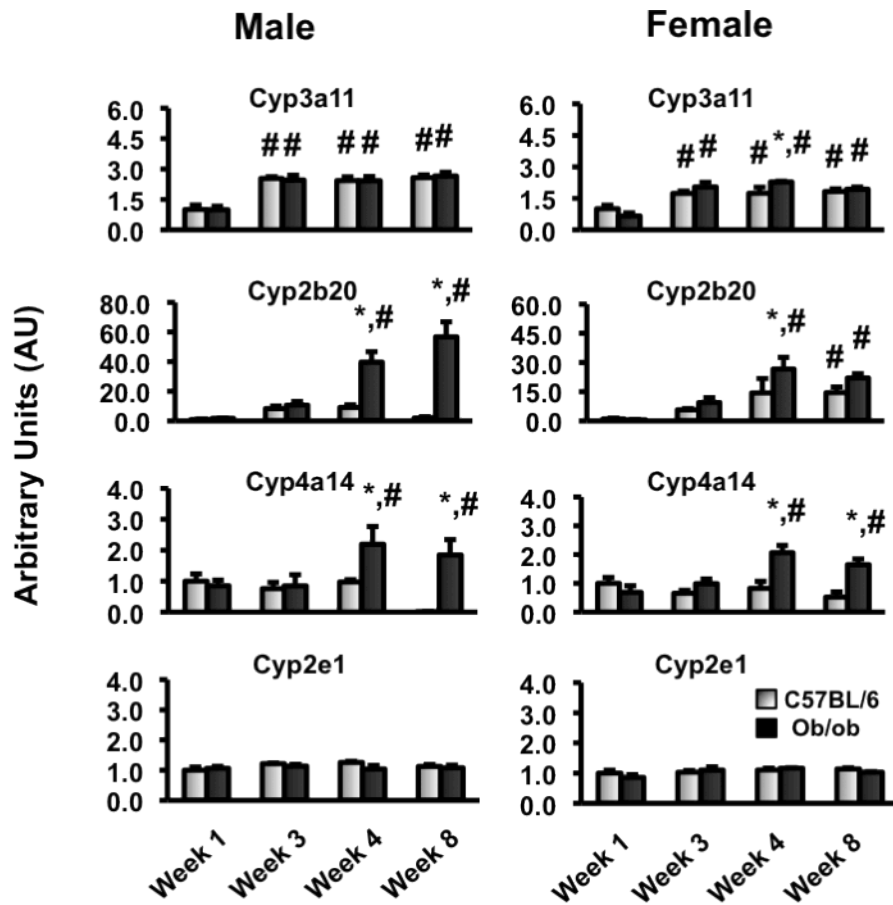


Figure 4. Effect of age and leptin deficiency on hepatic drug metabolizing enzyme (DME's) expression in both C57BL/6 and ob/ob mice

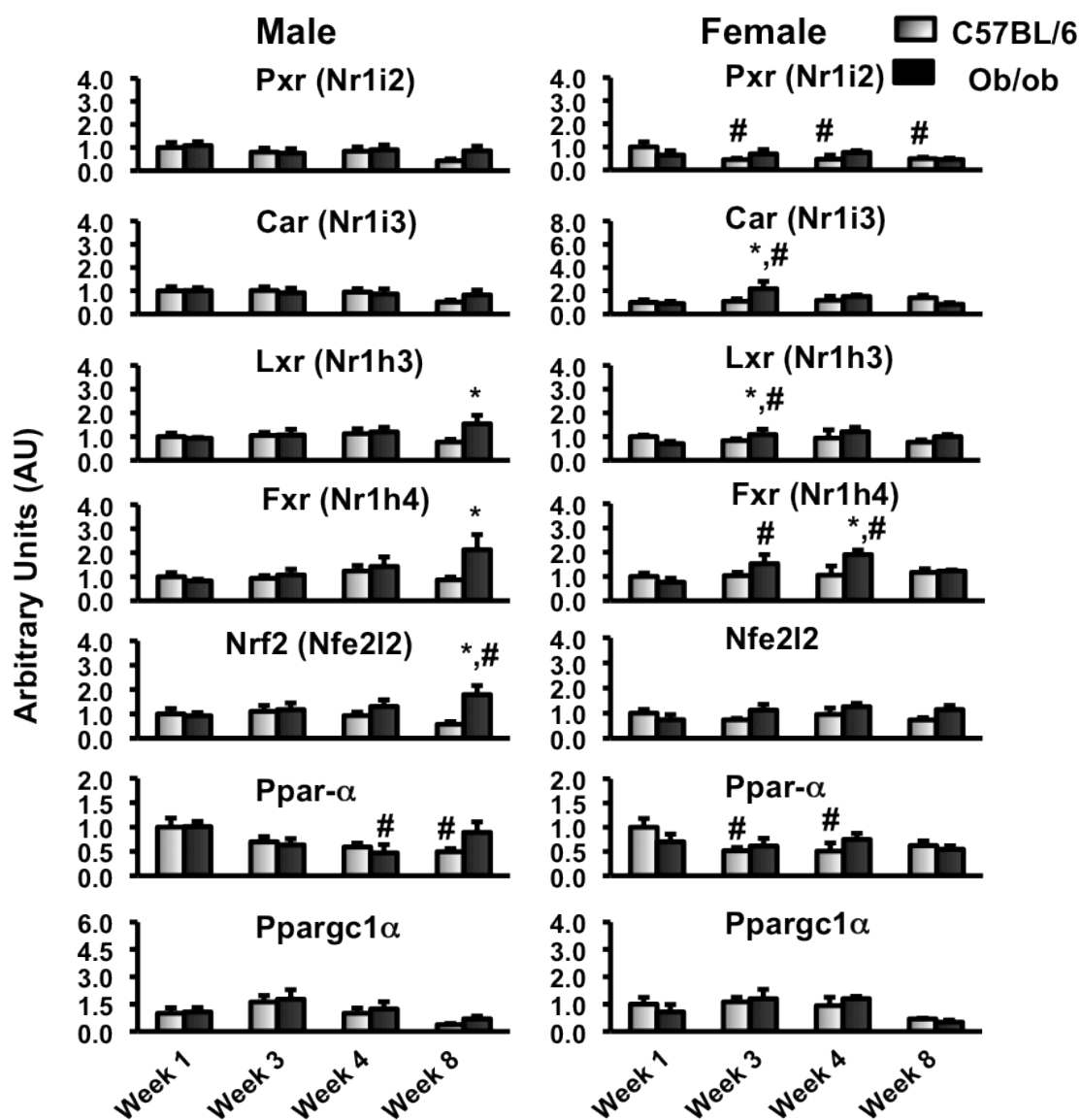


Figure 5. Effect of age and leptin deficiency on hepatic transcription factors expression in both C57BL/6 and ob/ob mice.

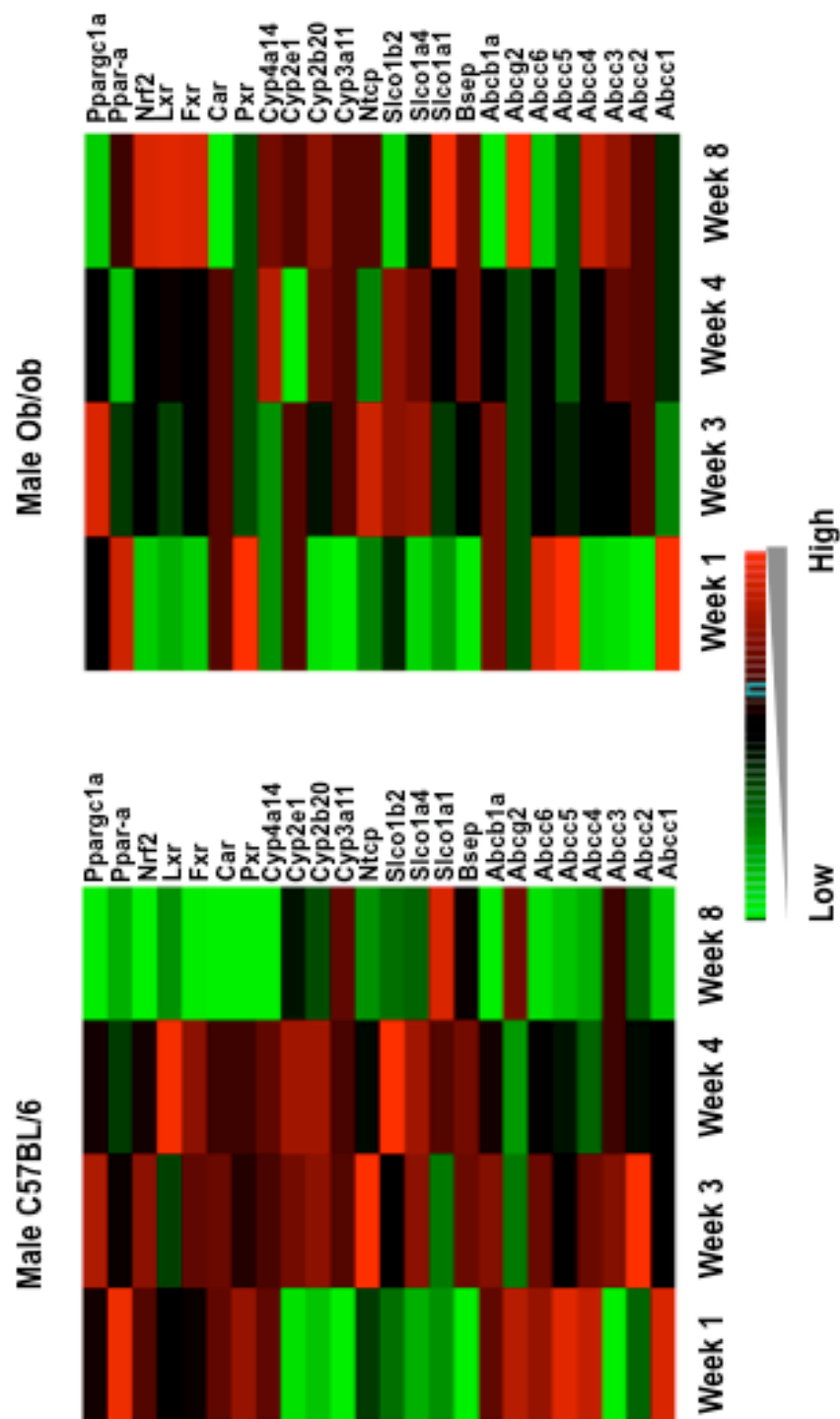


Figure 6. Heat maps for gene expression analysis performed in both male C57BL/6 and ob/ob mice

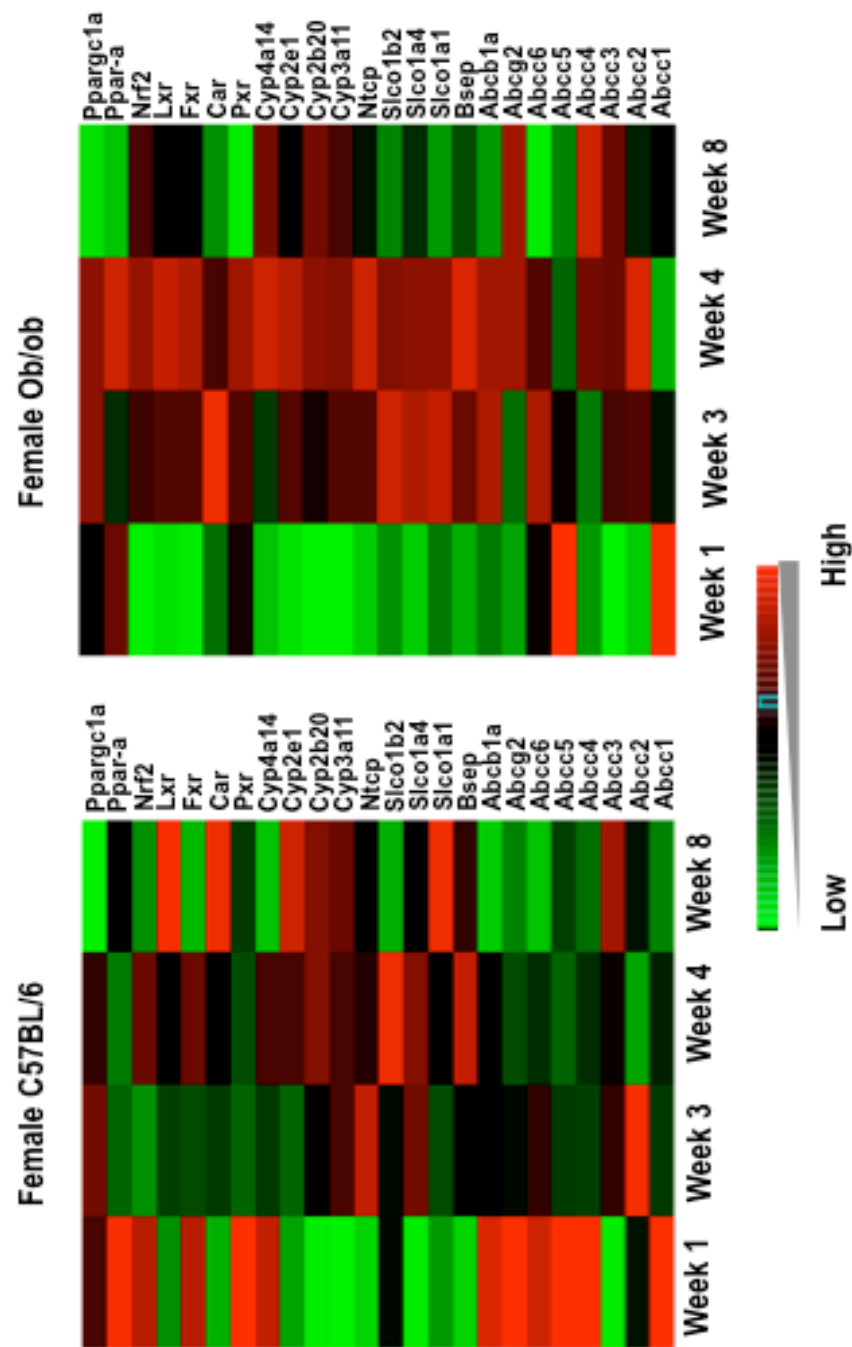


Figure 7. Heat maps for gene expression analysis performed in both female C57BL/6 and ob/ob mice

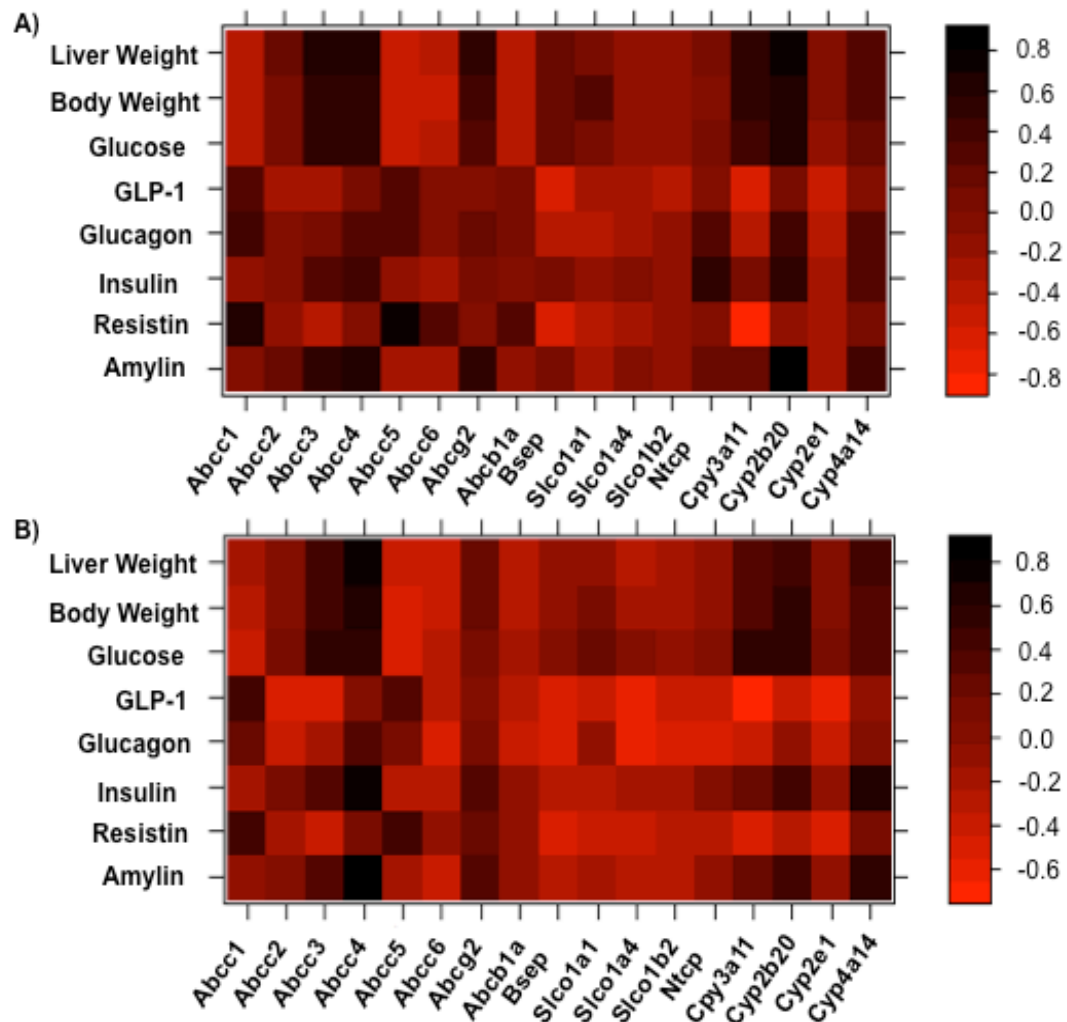


Figure 8. Correlation (heatmaps) between physiological factors with hepatic drug transporters and DME's expression during development of obesity in male (A) and female (B) mice.

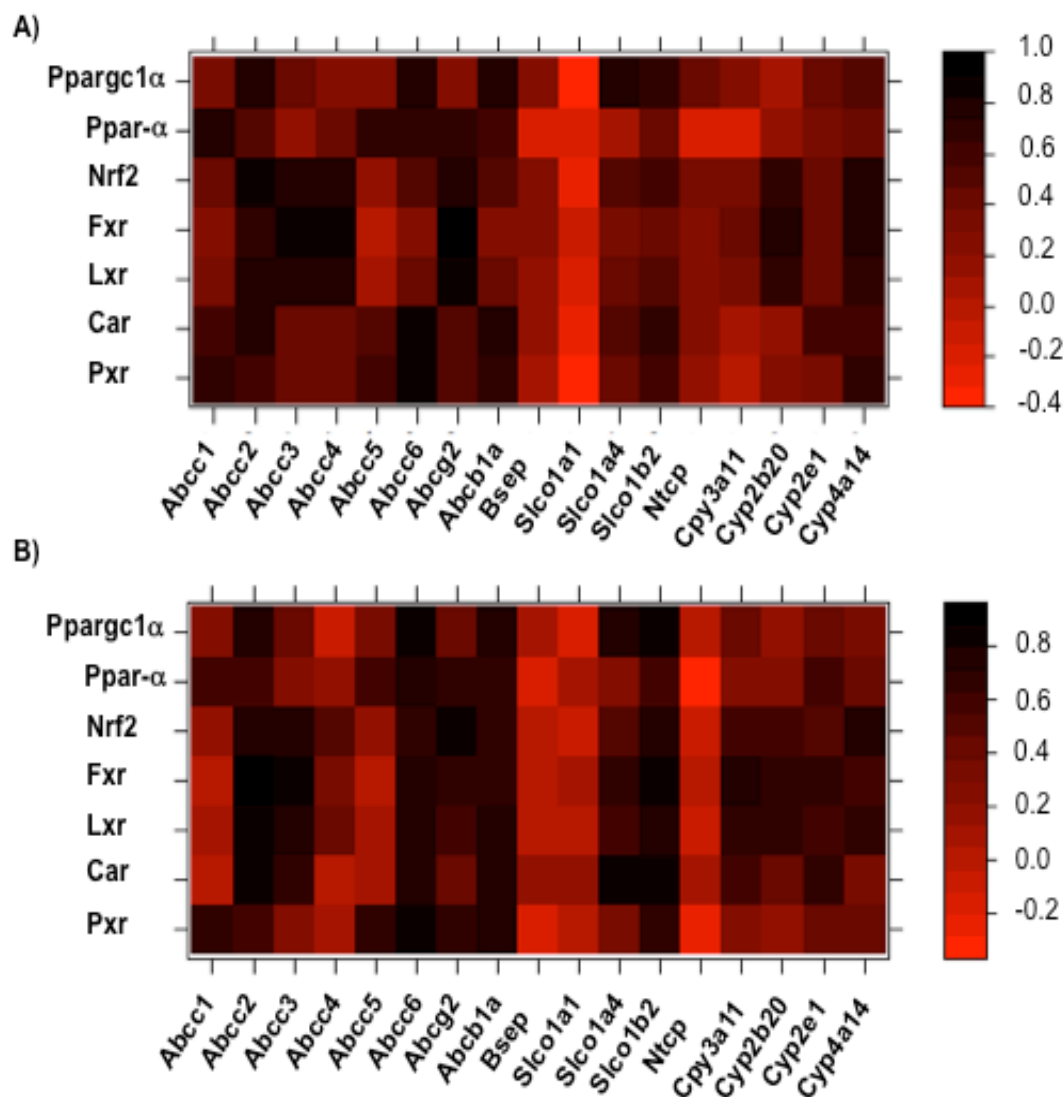


Figure 9. Correlation (heatmaps) between transcription factors with drug transporters and DME's expression during development of obesity in male (A) and female (B) mice.

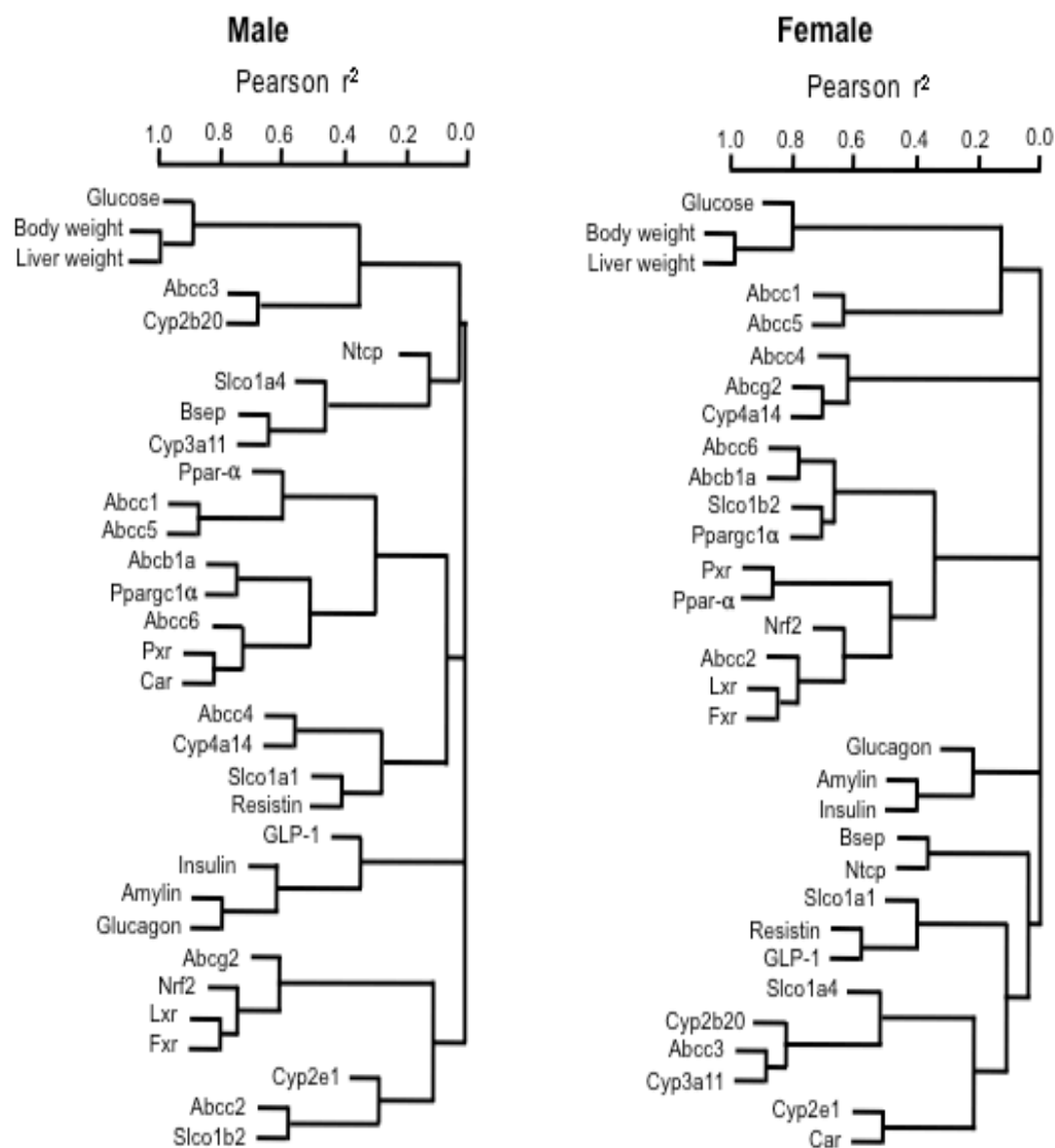


Figure 10. Hierarchical clustering of log transformed variables using Pearson correlation as a similarity measure.

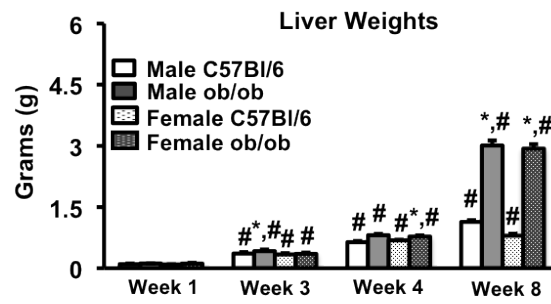


Figure 11. Effect of age and leptin deficiency on liver weights of C57BL/6 and ob/ob mice

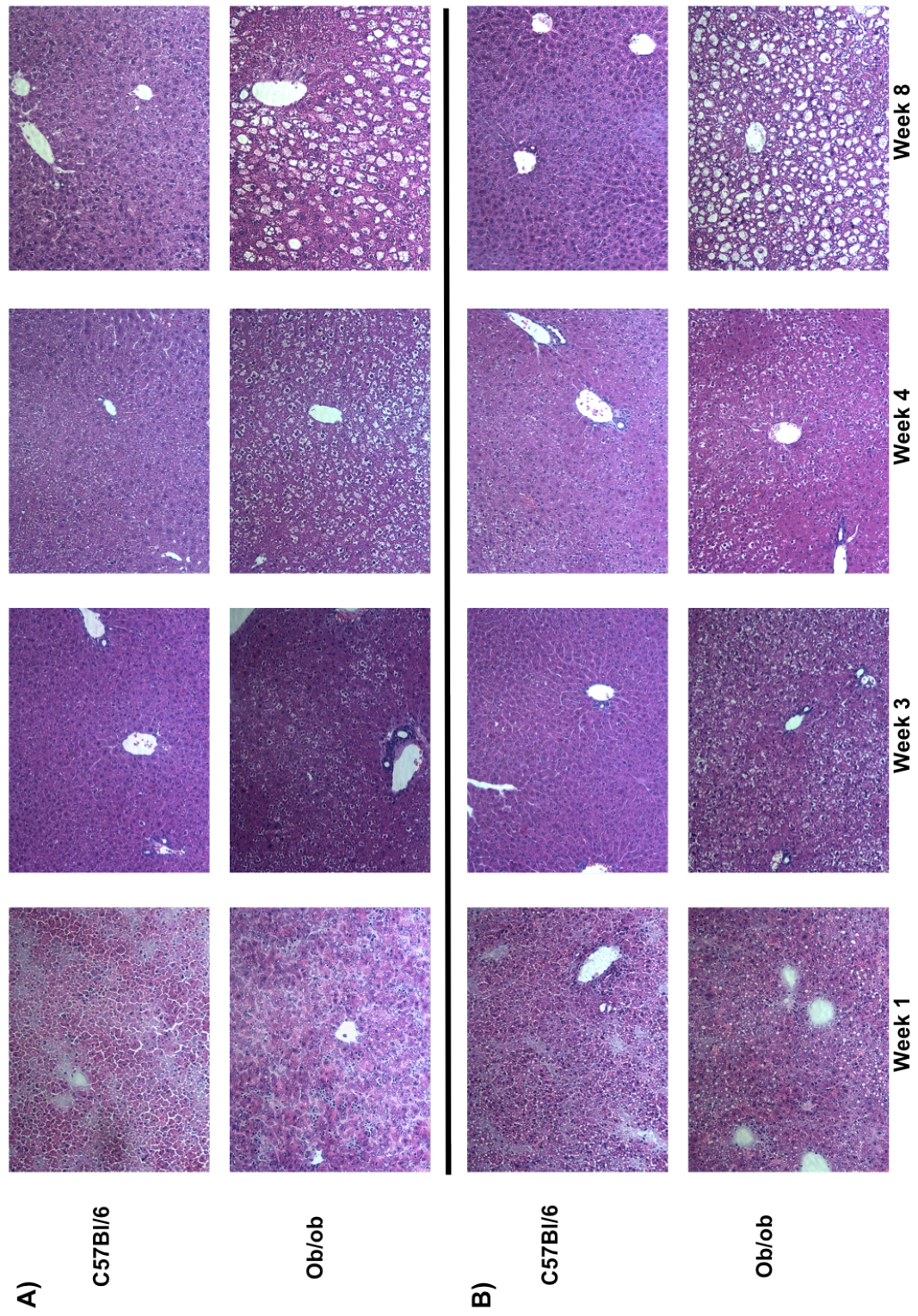


Figure 12. Effect of age and leptin deficiency on liver pathology.

Table 1. Correlation matrices for variables in phenotypical factors during development of obesity in male mice. Data presented as r values, * value indicates significant correlation ($p \leq 0.05$).

Male	Amylin	Resistin	Insulin	Glucagon	GLP-1	Glucose	Body weight
Resistin	0.46						
Insulin	0.86*	0.39*					
Glucagon	0.88*	0.75*	0.77*				
GLP-1	0.59*	0.55*	0.57*	0.72*			
Glucose	0.33	-0.45*	0.4*	0.02	-0.05		
Body weight	0.23	-0.53*	0.35*	-0.11	-0.13	0.94*	
Liver weight	0.23	-0.52*	0.36*	-0.12	-0.15	0.93*	0.99*

Table 2. Correlation matrices for variables in phenotypical factors during development of obesity in female mice. Data presented as r values, * value indicates significant correlation ($p \leq 0.05$).

Female	Amylin	Resistin	Insulin	Glucagon	GLP-1	Glucose	Body weight
Resistin	0.35						
Insulin	0.62*	0.57*					
Glucagon	0.46*	0.57*	0.52*				
GLP-1	0.37*	0.75*	0.52*	0.53*			
Glucose	0.42*	-0.37*	0.25	0.13	-0.28		
Body weight	0.47*	-0.38*	0.33	0.15	-0.21	0.91*	
Liver weight	0.46*	-0.34	0.38*	0.12	-0.14	0.88*	0.99*

Table 3. Correlation of hepatic transcription factors expression with hepatic drug transporters and DME's expression in adult (week-8 old) male mice. Data presented as r values, * value indicates significant correlation ($p \leq 0.05$).

<i>Male</i>	Pxr	Car	Lxr	Fxr	Nrf2	Ppar- α	Ppargc1a
Abcc1	0.95*	0.97*	0.98*	0.98*	0.93*	0.99*	0.95*
Abcc2	0.90*	0.98*	0.97*	0.92*	0.89*	0.97*	0.96*
Abcc3	0.96*	0.91*	0.93*	0.97*	0.95*	0.94*	0.94*
Abcc4	0.91*	0.83*	0.90*	0.93*	0.91*	0.87*	0.89*
Abcc5	0.89*	0.96*	0.93*	0.91*	0.86*	0.95*	0.91*
Abcc6	0.65*	0.73*	0.62	0.58	0.51	0.65*	0.68*
Abcg2	0.97*	0.97*	0.97*	0.99*	0.93*	0.98*	0.96*
Abcb1a	0.81*	0.71*	0.81*	0.80*	0.85*	0.76*	0.80*
Bsep	0.01	0.11	-0.02	-0.02	0.08	0.03	-0.01
Slco1a1	-0.35	-0.15	-0.33	-0.39	-0.49	-0.29	-0.27
Slco1a4	0.91*	0.97*	0.99*	0.93*	0.93*	0.98*	0.96*
Slco1b2	0.28	0.50	0.39	0.30	0.30	0.41	0.43
Ntcp	0.47	0.30	0.26	0.42	0.44	0.28	0.28

Table 4. Correlation of hepatic transcription factors expression with hepatic drug transporters and DME's expression in adult (week-8 old) female mice. Data presented as r values, * value indicates significant correlation ($p \leq 0.05$).

<i>Female</i>	Pxr	Car	Lxr	Fxr	Nrf2	Ppar- α	Ppargc1a
Abcc1	-0.60	-0.68*	-0.05	-0.30	-0.05	-0.61	-0.62
Abcc2	0.64	0.57	0.68*	0.48	0.60	0.79*	0.49
Abcc3	0.25	-0.28	0.81*	0.49	0.89*	0.23	0.07
Abcc4	-0.02	-0.54	0.67*	0.20	0.76*	-0.08	-0.28
Abcc5	0.17	0.35	0.24	0.42	0.20	0.42	0.67*
Abcc6	0.66	0.94*	0.03	0.18	-0.09	0.76*	0.63
Abcg2	0.21	-0.39	0.79*	0.41	0.81*	0.11	-0.19
Abcb1a	0.82*	0.52	0.61	0.28	0.48	0.72*	0.48
Bsep	0.09	0.35	-0.35	0.11	-0.38	0.15	0.41
Slco1a1	0.48	0.86*	-0.22	0.27	-0.43	0.59	0.39
Slco1a4	0.18	0.38	-0.14	0.33	-0.20	0.29	0.55
Slco1b2	0.65	0.63	0.51	0.72	0.46	0.82*	0.82*
Ntcp	0.31	0.35	-0.22	-0.27	-0.17	0.15	0.30

MANUSCRIPT II

Developmental BPA exposure impairs hepatic clearance mechanisms in adult mice via nuclear receptor down regulation and increased Hdac recruitment

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ABSTRACT

BPA is a synthetic xenoestrogen used in plastic manufacturing. Multiple studies in rodents demonstrate that developmental BPA exposure results in physiological, behavioral, and gene expression changes through multiple mechanisms – including epigenetic modifications. However, the impact of developmental BPA exposure on liver function was less known; as liver being a crucial determinant for systemic hormone concentrations and, chemical detoxification and clearance. The purpose of this study was to investigate the effect of developmental BPA exposure on the expression of hepatic Phase-II and Abc transporter expression involved in liver clearance processes and BPA metabolism. Female C57BL/6 (a/a) mice were fed either low or high dose of BPA (50 µg or 50mg/kg diet) or ethinyl estradiol (EE) (0.1ug/kg diet) or BPA (50mg/kg diet) with genistein (250 mg/kg diet) or AIN93 Control diet with 7% corn oil from 2 weeks pre-mating with male A^{vy/a} mice. BPA exposure was maintained through the breeding and lactation period. Livers were collected from male offsprings after PND 135. Developmental exposure to BPA and EE downregulated hepatic drug transporters and Phase II enzyme expression, involved in BPA metabolism and excretion whereas, genistein co-administration reversed these changes. Decrease in Nrf2 mRNA expression and binding activity could be partially responsible for downregulation of hepatic Phase II enzymes and drug transporter expression. Further, increase in expression of Hdacs upon BPA exposure could be responsible for decreased transcription factor expression and activity. Our data suggest that

developmental BPA and EE exposure may work via similar pathways, and greatly affect the expression of key hepatic genes involved in BPA and hormone metabolism and clearance.

INTRODUCTION

The liver is the major organ for metabolism and clearance; with a significant role in determining circulating systemic endocrine hormone and bile acid concentrations. Hepatic clearance of chemicals (e.g. hormones, drugs, and chemicals of exposure) relies upon several families of membrane-associated transport proteins working in concert with Phase-I and –II biotransformation enzymes. Numerous enzymes including Cytochrome P450s, UDP-glucuronosyl transferases (Ugts) and sulfotransferases (STs) are involved in biotransformation processes in liver. The resulting glucuronide and sulfate metabolites typically undergo fecal and urinary excretion through bile or blood via transporter-dependent mechanisms localized to the canalicular or basolateral membrane of hepatocytes, respectively.

Transporters are membrane proteins, which facilitate chemical transport into and out of cells (Klaassen and Aleksunes, 2010). In liver, the ATP-binding cassette (Abc) superfamily of transporters are involved in excretion of endogenous and xenobiotic compounds from the body, as well as enterohepatic recirculation of bile acids. Membrane transporter proteins are crucial in facilitating the uptake and biliary excretion of endogenous chemicals (e.g. conjugated hormones, bile acids, and conjugated bilirubin), and xenobiotics (e.g. environmental chemicals and drugs) (Faber *et al.*, 2003). Changes in certain transporters can cause imbalance in endogenous chemicals, such as bile acids, endocrine hormones, and bilirubin (Lecureux *et al.*, 2009). Moreover, xenobiotic metabolism is considered to be less efficient

without drug transporters (Faber et al., 2003). Impairment of certain drug transporters function, such as Multidrug resistance-associated protein 2 (Mrp2, Abcc2) and Bile salt-export pump (Bsep, Abcb11), cause hyperbilirubinemia and cholestasis respectively (Faber et al., 2003). During different pathological and physiological conditions, such as cholestasis or acetaminophen-induced liver injury, Abc transporter expression in liver is altered (Slitt et al., 2007; Aleksunes et al., 2008).

Bisphenol A (BPA) is a monomer used in plastic manufacturing. According to National Health and Nutrition Examination Survey, 92.6% of 2500 participants have BPA in urine samples (Calafat *et al.*, 2008). Perinatal BPA exposure to A^{vy} mice disrupted sexual dimorphic gene expression and sexual trait expression in adult mice (Mao et al., 2010; Jasarevic et al., 2011). *In utero* BPA exposure increased body weights in females altered mammary and reproductive glands, changed metabolic features, caused insulin resistance, increased adipogenesis, and predisposed to metabolic syndrome in pups (Alonso-Magdalena et al., 2006; Somm et al., 2009; Wei et al., 2011). BPA exposure is emerging as a well-studied example of the “fetal basis of disease”. The fetal basis of adult disease hypothesis proposes that various factors, such as nutrition or environmental exposure during prenatal and early postnatal development can affect development, resulting in susceptibility to chronic disease as an adult (Dolinoy et al., 2007b). Adverse effects elicited by developmental BPA exposure involve epigenetic mechanisms through modulation of DNA and histone methylation and endocrine disruption

(Vandenberg et al., 2009; Kundakovic and Champagne, 2011). For example, BPA perinatal exposure in A^{vy} mice changed the coat color of pups through epigenetic modulation via DNA hypomethylation, which was dampened by genestein supplementation along with BPA exposure (Dolinoy et al., 2007a). BPA also acts as an endocrine disruptor by mimicking estrogen and binds to estrogen receptors (ER, such as ER-alpha and beta. BPA is also known to act as a thyroid hormone antagonist (Moriyama *et al.*, 2002).

BPA is highly metabolized in liver and eliminated by urinary and fecal excretion (Pottenger et al., 2000; Volkel et al., 2002). BPA glucuronide (BPA-Gluc) and -sulfate (BPA-S) are the major metabolites formed in humans and mice (Pottenger et al., 2000; Volkel et al., 2002). The UGT2B family and SULT1A1 enzymes are major biotransformation enzymes involved in BPA conjugation (Nishiyama et al., 2002; Hanioka et al., 2008). In rodents, Abcc2 is the predominant transporter, which mediates BPA-Gluc excretion from liver into bile (Inoue *et al.*, 2005). Maternal exposure to other endocrine disruptors, such as polybrominated diphenyls (PBDEs) and BDE47 alters expression of metabolic enzymes and ABC transporters (Richardson et al., 2008; Szabo et al., 2009).

This study aimed to identify whether developmental BPA exposure could affect hepatic clearance processes, such as metabolism and disposition, in adulthood. In this study two different doses of BPA were administered; 50 µg/kg diet and 50 mg/kg diet (equivalent to 6.5 µg/kg body weight and 6.5 mg/kg bodyweight respectively) (Rosenfeld *et al.*, 2013). Along with BPA

exposure, dams were also exposed to ethinyl estradiol as a positive control or co-administrated genestein with BPA as previously described (Dolinoy et al., 2007a; Rosenfeld et al., 2013). Overall, the results herein detail that maternal exposure to BPA during gestation and lactation decreased hepatic transporter expression in male offsprings that were 135 days or older, which was in association with decreased recruitment of Hdac to the Abbc2 and 3 promoter.

MATERIALS AND METHODS

Animals and treatments: All animal experiments including breeding and dietary exposure were conducted by Dr. Cheryl Rosenfeld's laboratory at University of Missouri Columbia according to the IACUC regulations at the University of Missouri Columbia as previously described (Rosenfeld et al., 2013). Livers from this study (Rosenfeld et al., 2013) were used for the study herein.. Animal exposure and breeding was performed as previously described by Rosenfeld et al., 2013 (Rosenfeld et al., 2013). Briefly, virgin females (C57Bl/6, a/a) were fed one of the following five diets 1) Corn oil (control), 2) BPA 50 μ g/ Kg diet (low dose), 3) BPA 50mg/Kg diet (high dose), 4) Ethniyl estradiol (EE), 0.1 μ g/ Kg diet, 5) BPA 50mg/Kg and Genistein 250mg/Kg diet (BPA-G). Dams were fed the diet through gestation and lactation period. Pups were weaned and fed control diet, such that they were exposed to BPA only during the period of gestation and lactation. Livers were collected from male pups after postnatal day 135 days (n=5-6 per group).

RNA Isolation and cDNA Synthesis: Total RNA was isolated with RNAzol B reagent (Tel-Test Inc., Friendswood, TX). RNA concentration was assessed using NanoDrop™ (UV absorbance was measured at 260 nm) and the integrity was confirmed by formaldehyde gel electrophoresis. The mRNA samples were stored at -80°C until further use for analysis. cDNA for each sample was synthesized from 1 μ g of total RNA using cDNA synthesis Kit according to the manufacturer's protocol (Roche®, Germany).

qPCR Analysis: Gene expression was analyzed using Real-time quantitative PCR. qPCR was carried out using SYBRH Green PCR Master Mix (Roche®, Germany) with a Light Cycler 480 II (Roche®, Germany). Details of primers used were mentioned in Table 1.

Microsomal preparation: 200mg of liver tissue (n=3-6 per group) was washed with ice-cold saline and 50mM sodium pyrophosphate buffer (pH-7.4). Following washing livers were homogenized in 600 µl homogenizing buffer (0.25M Sucrose, 50mM Tris-Hcl (pH-7.8), 0.5mM EDTA, 20µM BHT, 0.1mM DTT) and centrifuged at 10,000x g for 15 min. The supernatant were centrifuged again at 100,000x g for 60 min. The resulting cytosols were stored at -80°C for future use. The microsomal pellet was re-suspended in buffer (0.25M Sucrose, 10mM Tris-Hcl (pH-7.8), 1mM EDTA, 20µM BHT and 20% glycerol) and stored at -80°C for future use.

Enzymatic BPA Glucuronidation and analysis: Hepatic microsomal fractions (0.0075mg) were incubated with 1µM BPA and 2mM UDPGA in 50mM phosphate buffer (pH-7.0) for 30 min at 37°C. At the end of Incubation, tubes were heated in boiling water to deactivate protein and centrifuged at 14000Xg for 10 min. Supernatant were collected and injected in to HPLC. BPA and BPA-Gluc were analyzed using HPLC-UV (System Gold®, Beckman Coulter, Inc.). 50 µl of each sample was injected onto column (ZORBAX SB-Aq, 5µm, 4.6X150mm, Agilent Technologies). A gradient elution used with 80% ACN and 20% 20mM phosphate buffer (pH-2.7) at 1 ml/min flow rate (Wen *et al.*, 2013) and analyzed at 200nm using an system Gold® UV detector

168 (Beckman Coulter, Inc). BPA and BPA-Gluc were identified using standards of BPA (Sigma-Aldrich) and BPA-Gluc (Toronto research chemicals, Canada) made in 50mM phosphate buffer (pH-7.0). BPA-Gluc and BPA are eluted at 7.4 and 10 minutes respectively. BPA and BPA-Gluc were quantified by comparing area of peak with BPA and BPA-Gluc standard curves (Wen et al., 2013).

Nuclear protein extraction: Nuclear extracts were isolated from liver tissue using Pierce NE-PER[®] nuclear extraction kit (Thermo Fisher Scientific, Rockford, IL, USA) and protein concentration was determined by the DC protein assay (Bio-Rad, Inc.).

Western Blotting: Western blots for transporters and nuclear histone deacetylase (Hdac), were performed according to previously published methods (Aleksunes et al., 2008; Donepudi et al., 2012). 50 µg nuclear, cytosolic, or crude membrane protein was loaded per well, and proteins were electrophoretically separated by a 4-20% SDS PAGE gel and transblotted onto PVDF membrane. Blots were blocked with 5% non-fat dry milk in TBST. The blots were incubated with primary antibody overnight, washed in TBST, and then incubated with secondary antibody for an hour. Staining was visualized by autoradiography. Antibodies details are Abcc2 (M₂III-5, Enzo Life Sciences, Farmingdale, NY), Abcc3 (M₃II-2), Abcc4 (M₄I-10), Abcg5 (BXP-53) from G. Scheffer laboratory (VU Medical Center, Amsterdam, The Netherlands), Bsep (K44), Ntcp (K4) from B. Steiger laboratory (University Hospital, Zurich, Switzerland), Hdac and Gadph (Cell signaling, MA, USA).

Transcription factor binding assay: Nuclear extracts obtained from livers were quantified for transcription factor binding to a prototypical ARE consensus sequence using a Procarta TF 9-plex custom array (Affymetrix, CA) according to the manufacturer's instructions and quantified using a Biorad Bioplex, which utilizes a luminex platform (Xu *et al.*, 2012a).

Hepatic bile acid levels: Hepatic bile acids were extracted from using tertiary-butanol extraction method and quantified as described previously (Donepudi *et al.*, 2012) using a bile acid assay kit (Bioquant, San Diego, CA, USA).

Hepatic Glutathione levels: Hepatic reduced glutathione (GSH) levels were measured using GSH-GloTM Glutathione assay kit (Promega). Briefly, 20mg of liver tissue was homogenized in 2ml of 2mM EDTA in phosphate buffered saline (PBS) and homogenate was centrifuged. The resulting supernatant was analyzed for hepatic glutathione levels. Hepatic GSH levels were analyzed according to manufacturer's protocol. Luminescence was measured using a GloRunnerTM microplate luminometer interfaced with GloRunner DXL Software (Turner Biosystems, Sunnyvale, CA, USA).

Dibromosulfophthalein (DBSP) disposition: DBSP (120mmole/kg/5ml, *i.p.*) was injected into mice. Mice were euthanized 45 min after DBSP injection and DBSP concentration was determined in gallbladder bile. Concentration of DBSP was quantified spectrophotometrically at 575 nm after alkalization of the samples with 0.1M NaOH.

Statistical Analysis: The statistical significance between groups was

determined using one-way ANOVA followed by a Duncan's *post hoc* test, using Statistica 9.1 software (Stat Soft Inc., Tulsa, OK, USA). Data are presented as mean \pm SE, with $P \leq 0.05$ considered statistically significant.

RESULTS

Effect of developmental exposure of BPA on Body and liver weights.

Figure 1 illustrates body and liver weights of male pups, which have undergone developmental exposure of BPA, EE and BPA-G. Body weights were similar among BPA, EE and BPA-G developmental exposure in pups. Similar to body weights, exposure to the various diets did not change the liver weights.

Effect of developmental exposure of BPA on Phase-II conjugation.

Figure 2A illustrates the effect of developmental BPA exposure on hepatic BPA glucuronidation. In rodents, Ugt2b1 and Sul1a1 are Phase-II enzymes involved in BPA metabolism (Yokota et al., 1999; Nishiyama et al., 2002). Both BPA and EE developmental exposure decreased Ugt2b1 expression in pups to 35 and 31%, and Sult2a1 mRNA levels by 48 and 27 % of control diet-exposed pups respectively. BPA-Gluc is the predominant BPA metabolite formed by humans and rodents, formed through glucuronidation (Yokota et al., 1999). Figure 2B illustrates the effect of BPA developmental exposure on hepatic BPA glucuronidation capacity at a relatively low BPA concentration. Similar to mRNA levels of Ugt2b1, BPA glucuronidation levels decreased with BPA developmental exposure. BPA high dose and EE developmental exposure decreased BPA glucuronidation in pups to about 74% and 81% of control, respectively. Genistein co-administration reversed the observed decrease caused by BPA developmental exposure. The BPA concentration (1 μ m) in the reaction vessels was chosen because it is a more relevant

concentration and lower or as low as previously described (Wen et al., 2013). The observed percent decrease in BPA glucuronidation is less than the observed decrease in Ugt2b1 expression, and this is likely due to the low concentration of BPA used, which is less than K_m (Mazur et al., 2010).

Effect of developmental exposure to BPA on hepatic basolateral drug transporter expression. Figure 3 illustrates effect of BPA developmental exposure on hepatic basolateral drug transporter expression in pups. Hepatic basolateral drug transporters such as Abcc3, 4, and Ntcp expression decreased with BPA developmental exposure. These observed changes were similar to EE exposure, whereas the effects caused by BPA developmental exposure were not observed with genistein exposure. In pups, with both doses of BPA exposure Abcc4 and Ntcp mRNA levels decreased about to 21 and 31 % of controls, whereas Abcc3 mRNA levels in livers of adult sons had a decreasing trend with BPA exposure. Developmental exposure to EE decreased Abcc3, 4, and Ntcp mRNA levels about to 21, 2 and 26 %, of controls respectively. Similar to mRNA levels, developmental BPA exposure decreased protein expression of Abcc3 and 4. Ntcp protein expression decreased with both doses of BPA and increased with EE exposure compared to the control diet group despite decreased mRNA levels.

Effect of developmental exposure to BPA on apical drug transporter expression. Figure. 4 illustrates the effect of BPA developmental exposure on expression of canalicular drug transporter expression in livers of adult male sons. In livers of adult male sons, with both doses of BPA exposure Abcc2,

Abcg2, and Bsep mRNA levels were decreased to 54, 40 and 41 % of control diet-exposed group respectively. EE developmental exposure decreased Abcc2, Abcg2, and Bsep mRNA levels about to 36, 20 and 22% of control diet group respectively. Similar to mRNA levels, BPA developmental exposure decreased Abcc2 protein expression in liver. Abcg2 protein expression was similar to controls and unaltered with BPA and EE exposure, EE exposure increased Bsep protein expression, whereas Bsep expression was similar between BPA exposed and control groups.

Developmental BPA exposure perturbs bile acid and GSH levels in liver.

Hepatic Abc transporters are determinants for the excretion and enterohepatic circulation of bile acids. Altered hepatic Abcc2 expression was observed during cholestasis (Geier *et al.*, 2003), a pathological condition caused by impendence of bile acids leading to bile acid accumulation in liver. Exposure to the low BPA dose significantly increased hepatic bile acid concentration by 30% compared to controls, but not at the higher BPA dose. Abcc2 transports utilizes GSH as cofactor to drive biliary excretion. Moreover Abcc2^{-/-} mice have increased hepatic total glutathione levels (Chu *et al.*, 2006). BPA exposure increased GSH levels in liver by 60 and 80%, respectively, compared to concentrations on livers of controls (Figure 5B).

Effect of developmental exposure of BPA on DBSP disposition. DBSP disposition is used as a estimation of hepatic transport function (Dhumeaux *et al.*, 1974) and targeted Abcc2 deletion significantly decreased DBSP levels in mice (Chu *et al.*, 2006), illustrating DBSP as a substrate for mouse Abcc2.

Both BPA exposures significantly reduced DBSP levels in bile to 24 and 33%, of control diet group respectively (Figure 5C). The observed decrease in DBSP concentration in gallbladder bile likely reflects the corresponding decrease in liver Abcc2 expression.

Developmental BPA exposure decreases transcription factor expression and binding. Figure 6 illustrates the effect of developmental BPA exposure on gene expression and binding activity of transcription factors involved in regulation of drug transporters and Phase-II enzymes. In rodents, transcription factors such as Ahr, Car, Fxr and Nrf2 are known to regulate drug transporters and Phase-II enzyme expression (Rushmore and Kong, 2002; Nakata et al., 2006). BPA, at both concentrations, decreased Ahr, Car, Fxr and Nrf2 mRNA levels in livers of adult sons by at least 41, 36, 53 and 58%, respectively, which was similar to EE developmental exposure. Conversely, the decrease in hepatic transcription factor expression was not observed in livers from pups that had maternal dietary genistein supplementation. In contrast to mRNA expression, transcription factor such as Ahr, Car and Fxr binding activity was unaltered at the lower BPA concentration fed to dams. However, the higher BPA dose and EE developmental exposure decreased Ahr, Car, and Fxr binding activity. Both BPA exposures decreased Nrf2 binding, which was not observed in livers from pups from dams fed BPA with genestein.

Developmental BPA exposure increases Class 1 Hdac expression. In order to determine a mechanism by which decreased biotransformation,

transporter, and nuclear receptor expression occurs, it was hypothesized that developmental BPA exposure might affect pathways with panoramic effects of gene transcription. Hdacs are known to modulate or down regulate expression genes such transporters. Figure 7 illustrates Class I Hdac protein expression in nuclear extracts obtained from livers of adult sons from dams that were fed control, BPA, EE, or BPA-G diet. Class I Hdac proteins such, as Hdac 1, 2 and 3, nuclear levels was increased with both doses of BPA and EE developmental exposure compared to control group. Genistein co-administration slightly increased Hdac1, 2 and 3 nuclear levels but not to the level of BPA and EE developmental exposure groups.

Discussion:

BPA a xenoestrogen present ubiquitously in the environment (Vandenberg *et al.*, 2009), and is known to cause several adverse effects (Vandenberg *et al.*, 2009). BPA exposure in humans is variable as studies report different values of estimated human exposure (Vandenberg *et al.*, 2009). Toxicological studies identify 1000 mg BPA/kg body weight (BW)/day as maximum tolerable dose in humans (Vandenberg *et al.*, 2009). According to EPA, the daily tolerable intake or no observed adverse effect levels (NOAEL) of BPA is 50 µg/kg BW/ day (Vandenberg *et al.*, 2009). In this study, two concentrations BPA were supplemented in maternal feed, one below NOAEL, one higher than the NOAEL. Several studies documented, in rodent models, that BPA exposure is associated with physiological, behavioral and gene expression changes at or below NOAEL (Alonso-Magdalena *et al.*, 2006; Somm *et al.*, 2009; Jasarevic *et al.*, 2011; Wei *et al.*, 2011; Patisaul *et al.*, 2012), indicating BPA can causes several adverse effect below NOAEL.

Despite liver being important for elimination of endocrine disrupting compounds and determining systemic hormone levels, few studies address the effect of BPA on hepatic processes like metabolism. BPA undergoes extensive Phase-II conjugation, which is essential for decreasing BPA body burden (Volkel *et al.*, 2002) and conjugated BPA does not have endocrine disruption effects caused by BPA (Matthews *et al.*, 2001). BPA primarily undergoes glucuronidation, whereas sulfonation can occur, but is considered to have a minor, but appreciable role (Pottenger *et al.*, 2000). Species-specific

differences were observed in BPA elimination pathways. In rodents, BPA-Gluc primarily undergoes biliary excretion whereas in humans, BPA undergoes urinary excretion (Vandenberg et al., 2009). *In vitro* studies using ATPase assays have identified ABCC3 in humans as a possible drug transporter involved in transport of BPA-Gluc (Mazur *et al.*, 2012), whereas in rodents Abcc2 is major transporter of BPA-Gluc (Inoue et al., 2005).

In the present study, the effects of developmental exposure of BPA on liver function and hepatic metabolic processes in adult were documented. Developmental BPA exposure downregulated key enzymes and transporters involved in BPA metabolism and excretion. Developmental BPA exposure also decreased activity or function of genes involved in BPA metabolism and excretion. Effects observed in this study by developmental BPA exposure are similar to EE developmental exposure although there were no changes in estrogen levels in these mice (Figure 10). BPA and EE developmental exposure significantly down-regulated hepatic Phase II enzymes such as Ugt2b1 and Sult1a1, and hepatic efflux drug transporters such as Abcc2, 3, and 4 expressions. Developmental BPA and EE exposure also decreased BPA glucuronidation capacity of pups, which supports decrease in Phase II enzyme expression in liver. Decrease in hepatic efflux transporters, primarily Abcc2 expression was supported by increase total hepatic glutathione levels and decrease of DBSP levels in bile. As mentioned previously, glutathione conjugates and DBSP are substrates for Abcc2 (Chu et al., 2006). Interestingly, changes in hepatic gene expression such Phase II enzymes and

Abc transporter expression due to developmental BPA exposure was reversed by genistein co-administration. Serum BPA levels in dams were unaltered with genistein supplementation (Figure 8), indicating genistein co-administration did not change BPA exposure to dams and effects shown by genistein co-administration did not involve differences in BPA exposure. Decrease in hepatic metabolism genes and transcription factors by BPA developmental exposure in this study were supported by similar observation in CD-1 male pups, which are exposed to BPA at perinatal, and peripubertal stages (Figure 11).

Our results show, both doses of developmental BPA and EE exposure downregulated transcription factor expression such as Ahr, Car, Fxr, and Nrf2, and binding activity at high dose of BPA and EE developmental exposure. Only Nrf2 binding activity to its consensus sequence was decreased with both doses of BPA developmental exposure similar to Phase II and drug transporter expression changes caused by BPA. This indicates Nrf2 role in BPA or EE induced downregulation of hepatic drug transporters and Phase II enzymes in pups. Decrease in Nrf2 expression and activity was supported by decrease in its target gene Nqo1 expression (data not shown).

Hepatic Ugt2b1 is a key enzyme involved in conjugation of endogenous androgens and xenobiotics (Yokota et al., 1999). Although Phase II enzymes particularly Ugt2b1 expression is minimally induced with transcription factors activators, Nrf2-null mice showed significant decrease in Ugt2b1 expression indicating basal levels of Ugt2b1 expression is regulated by Nrf2 (Buckley and

Klaassen, 2009; Reisman et al., 2009). In mice, Sult1a1 expression is significantly upregulated by only butylated hydroxyanisole (BHA), which is an activator for Nrf2. In our study also decrease in Sult1a1 and Ugt2b1 expression is similar decrease in Nrf2 expression and binding activity indicating BPA induced downregulation of Phase II enzyme expression caused through repression of Nrf2 activity.

Studies have shown that hepatic efflux transporters such as Abcc2, 3 and 4 expression are regulated by several transcription factors such as Ahr, Car and Nrf2 (Maher et al., 2005). In mice, prototypical activators of these transcription factors increase hepatic efflux transporter expression (Maher et al., 2005). In mice, activation of Nrf2 by oxidative stress and chemical activators increased hepatic Abc transporters expression such as Abcc2, 3 and 4. Moreover, hepatic efflux transporters such as Abcc2, 3, 4 and Abcg2 expression decrease significantly in Nrf2-null mice whereas their expression significantly increased in Keap1 knock down and Keap1 hepatocyte knockout mice (Reisman et al., 2009; Wu et al., 2012). Similar to these studies, developmental BPA at both doses and EE exposure decreased hepatic efflux transporter expression and only Nrf2 expression and activity in same pattern, indicating Nrf2 role in BPA induced down regulation of hepatic Abcc2, 3 and 4 transporters.

In mice, developmental BPA exposure is known to induce epigenetic changes such as hypomethylation and histone modification in different tissues at different promoter regions (Kundakovic and Champagne, 2011). Epigenetic

changes specifically hypomethylation of intracisternal A particle (IAP) retrotransposon of agouti promoter region caused by developmental BPA exposure is reversed with methyl donor and genistein supplementation (Dolinoy et al., 2007a). Moreover, as mentioned earlier effects observed in this study by BPA are similar to EE indicating these changes are caused due to endocrine component involved in it which is known to cause epigenetic changes in pups (Vandenberg et al., 2009). In our study we observe a decrease in methyl cytosine levels in genomic DNA isolated from liver with developmental exposure of BPA although these changes are not reversed with genistein co-administration (Figure 9). Apart from DNA methylation, histone modification such as methylation and acetylation also can regulate nuclear receptor inducible gene transcription (Biddie, 2011). Hdac proteins are known to remodel chromatin structure and act as co-repressors for nuclear receptor inducible gene transcription (Biddie, 2011). Results presented in this study show that developmental BPA and EE exposure increased nuclear Hdac levels whereas genistein co-administration did not as much as BPA. Increase in class I Hdac nuclear levels indicate decrease in nuclear receptor inducible gene transcription. *In vitro* studies had shown role of class I Hdac proteins in regulation of efflux transporter expression (To et al., 2008; Xu et al., 2012b). Moreover studies have shown that Hdac proteins can regulate Nrf2 induced transcription and expression (Yu et al., 2010; Lee et al., 2012). In accordance to previous studies and our results, we think developmental BPA and EE exposure decrease hepatic gene expression by increasing Hdac levels and

decreasing nuclear receptor inducible gene transcription in pups.

Developmental BPA exposure decreases hepatic Phase II and drug transporter expression in pups similar to EE exposure and is reversed by genistein co-administration. The observations from current study indicate changes in Phase II enzymes and drug transporter expression due to developmental BPA exposure was due caused by partly due to epigenetic effects such as increase in class I Hdac nuclear levels and down regulation of transcription factors. Decrease in hepatic genes such as drug transporter and Phase II enzymes, which are involved in metabolism and elimination of endogenous hormones and xenobiotics can alter drug and hormonal metabolism. Most importantly altered hepatic gene expression that are involved in metabolism due developmental BPA exposure are persistent and are caused below and above NOAEL, which supports BPA can cause adverse effects at very low doses (Vandenberg et al., 2009). Hepatic Phase II and drug transporter that are altered with developmental exposure of BPA are also involved in hormone metabolism and elimination, changes in these gene expression might be responsible for endocrine disruption caused by BPA.

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Figure Legends

Figure 1. Effect of developmental BPA exposure on body and liver weights. Both BPA and EE developmental exposure did not affect body and liver weights of male pups. Data is presented as average weights (grams) \pm S.E.M. (n=5-6 mice per group). An asterisk (*) represents a statistical difference between pups from control diet fed dams to pups from BPA and EE exposed dams ($p \leq 0.05$).

Figure 2. Effect of developmental BPA exposure on Phase II enzymes, expression and activity, which are involved in BPA metabolism. Total RNA was isolated from livers of male pups that are developmentally exposed to either of diets such as control, BPA 50 μ g/kg and 50mg/kg, EE and BPA-G. (A) Hepatic phase II enzymes such as Ugt2b1 and Sult1a1 mRNA levels were quantified by quantitative PCR. Data was normalized to 18S rRNA and presented as a mean arbitrary units \pm S.E.M. (n=5-6 mice per group). (B) Enzymatic glucuronidation activity in male pups. Hepatic microsomes were isolated from frozen liver tissue. BPA glucuronidation assays were carried out with 1 μ M BPA and BPA glucuronide formed was analyzed using HPLC. Data presented as mean BPA glucuronide formed (nmoles/mg/min) \pm S.E.M. (n=3-7 mice per group). An asterisk (*) represents a statistical difference between pups from control diet fed dams to pups from BPA, EE and BPA-G exposed dams ($p \leq 0.05$).

Figure 3. Effect of developmental BPA exposure on hepatic basolateral transporter expression. Total RNA was isolated from livers of male pups

that are developmentally exposed to either of diets such as control, BPA 50µg/kg and 50mg/kg, EE and BPA-G. (A) Hepatic basolateral transporter such as Abcc3, 4 and Ntcp mRNA levels were quantified by quantitative PCR. Data was normalized to 18S rRNA and presented as a mean arbitrary units \pm S.E.M. (n=5-6 mice per group). An asterisk (*) represents a statistical difference between pups from control diet fed dams to pups from BPA, EE and BPA-G exposed dams ($p \leq 0.05$). (B) Protein quantification of hepatic basolateral drug transporters using western blots. Data presented in order of control, BPA 50µg/kg and 50mg/kg, EE and BPA-G diet and n=2 per group.

Figure 4. Effect of developmental BPA exposure on hepatic apical/canicular transporter expression. Total RNA was isolated from livers of male pups that are developmentally exposed to either of diets such as control, BPA 50µg/kg and 50mg/kg, EE and BPA-G. (A) Hepatic apical/canicular transporter such as Abccc2, Bsep (Abcb11) and Abcg2 mRNA levels were quantified by quantitative PCR. Data was normalized to 18S rRNA and presented as a mean arbitrary units \pm S.E.M. (n=5-6 mice per group). An asterisk (*) represents a statistical difference between pups from control diet fed dams to pups from BPA, EE and BPA-G exposed dams ($p \leq 0.05$). (B) Protein quantification of hepatic basolateral drug transporters using western blots. Data presented in order of control, BPA 50µg/kg and 50mg/kg, EE and BPA-G diet and n=2 per group.

Figure 5. Effect of developmental BPA exposure on hepatic clearance. Changes in endogenous and xenobiotic disposition caused due to

developmental BPA exposure in pups. (A) Hepatic bile acid levels in male pups from dams exposed to BPA. Hepatic bile acid were quantified and data was presented as average concentrations ($\mu\text{mole/l/mg}$) \pm S.E.M. (n=5-6 mice per group). (B) Hepatic total glutathione levels in male pups from dams exposed to BPA. Hepatic total glutathione levels were quantified and data was presented as average concentrations (nmole/mg) \pm S.E.M. (n=5-6 mice per group). (C) DBSP disposition in male pups from dams exposed to BPA. DBSP levels in gall bladder was analyzed and data presented as concentration (Molar/g of gall bladder) \pm S.E.M. (n=3-6 mice per group). An asterisk (*) represents a statistical difference between male pups from control diet fed dams to pups from BPA, exposed dams ($p \leq 0.05$) and (#) was indicated for statistical significance of $p = 0.06$.

Figure 6. Effect of developmental BPA exposure on transcriptional factors expression and binding activity. Total RNA was isolated from livers of male pups that are developmentally exposed to either of diets such as control, BPA 50 $\mu\text{g/kg}$ and 50mg/kg, EE and BPA-G. (A) Transcription factors such as Ahr, Car, Fxr and Nrf2 mRNA levels were quantified by quantitative PCR. Data was normalized to 18S rRNA and presented as a mean arbitrary units \pm S.E.M. (n=5-6 mice per group). (B) Transcription factor binding activity of Ahr, Car, Fxr and Nrf2 was analyzed using TF procarta assay. Data presented as mean multiple fluorescence index (MFI) for n=2 samples per group. An asterisk (*) represents a statistical difference between pups from

control diet fed dams to pups from BPA, EE and BPA-G exposed dams ($p \leq 0.05$).

Figure 7. Effect of developmental exposure of BPA on nuclear levels of class I Hdac proteins and Hdac recruitment. (A) Protein quantification of nuclear Hdac1, 2 and 3 levels was performed using western blots. Data presented in order of EE, control, BPA 50 μ g/kg and 50mg/kg, and BPA-G diet and $n=1$ per group. Developmental BPA and EE exposure increased nuclear levels of class I Hdac proteins in pups.

Figure 8. Serum BPA levels in dams exposed to different diets. Data was presented as average total BPA concentration (ng/ml) \pm S.E.M ($n=4-6$ mice per group). An asterisk (*) represents a statistical difference between pups from control diet fed dams to pups from BPA, EE and BPA-G exposed dams ($p \leq 0.05$).

Figure 9. Effects of developmental BPA exposure on global methylation in male pups livers. Total genomic DNA was isolated from livers and analyzed for methylated cytosine levels using methylation kits (Epigentech). Data was presented as average percentage of (5-methyl cytosine) \pm S.E.M ($n=5-6$ mice per group). An asterisk (*) represents a statistical difference between pups from control diet fed dams to pups from BPA and BPA-G exposed dams ($p \leq 0.05$).

Figure 10. Effects of developmental BPA exposure on serum estrogen levels in male pups. Serum estrogen levels are determined using serum

estrogen ELISA kit (Calbiotech, CA, USA). Data was presented as average concentration (pg/ml) \pm S.E.M (n=5-6 mice per group).

Figure 11. Effects of developmental BPA exposure on hepatic phase II and drug transporter expression in male CD-1 pups. Female CD-1 mice (12 weeks old) were bred at Tufts University Human Nutrition, Boston, MA. . On gestational day (GD) 6 dams were weighed and osmotic pumps (model 1004; Alza Corp., Palo Alto, CA, USA) with flow rate 0.11 μ l/hr were implanted into pregnant moms on GD 8 such that pups receive either vehicle (50% DMSO) or two BPA (25, 250 μ g/kg BW/ day) treatments. Pups are weaned and maintained on *ad libitum* diet. Post weaning pups were exposed to similar BPA doses through drinking water. Tissues from female offspring were collected on postnatal day 32. Total RNA was isolated from livers of male pups that are developmentally exposed to DMSO and BPA and mRNA levels were quantified by quantitative PCR. Data was normalized to 18S rRNA and presented as a mean arbitrary units \pm S.E.M. (n=9-10 mice per group). An asterisk (*) represents a statistical difference between pups from control to pups from BPA exposed dams ($p \leq 0.05$).

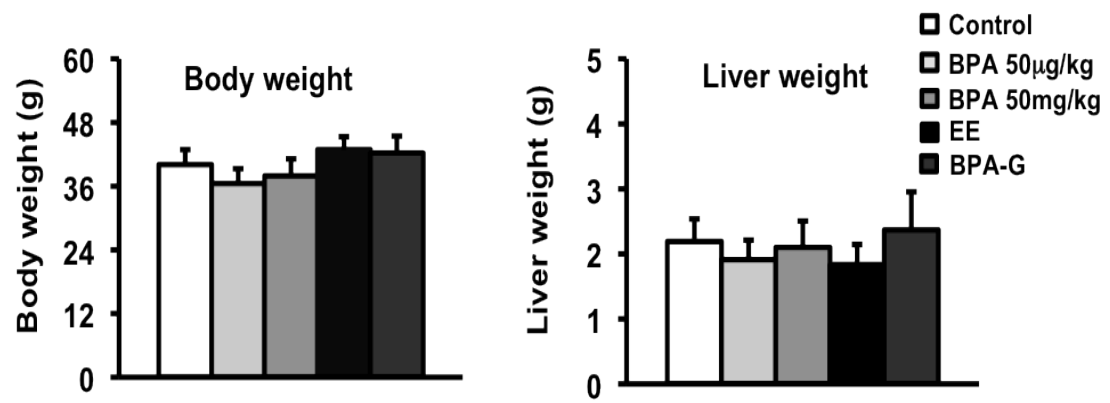
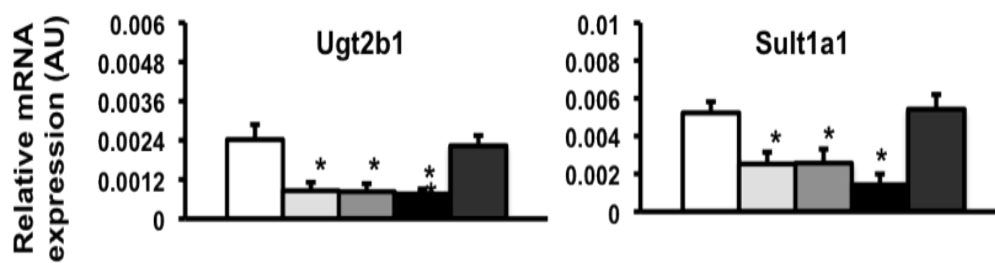


Figure 1. Effect of developmental BPA exposure on body and liver weights.

A)



B)

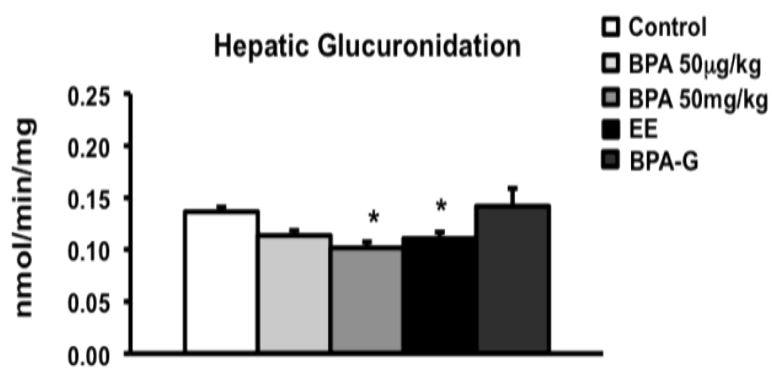


Figure 2. Effect of developmental BPA exposure on Phase II enzymes, expression and activity, which are involved in BPA metabolism.

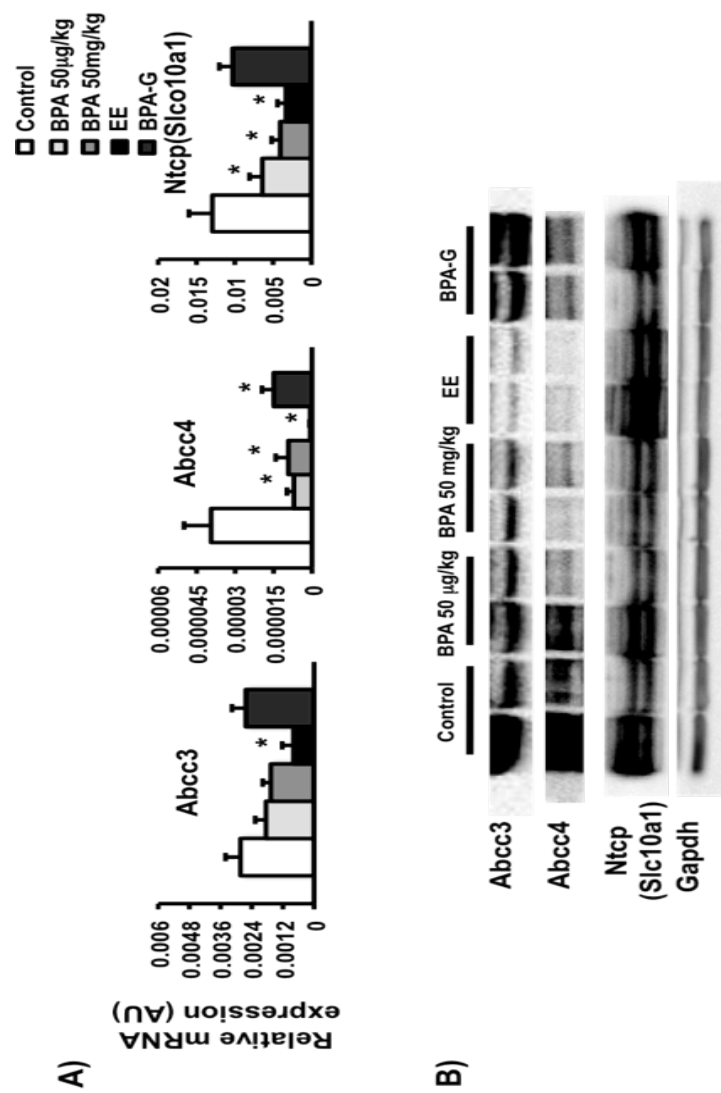


Figure 3. Effect of developmental BPA exposure on hepatic basolateral transporter expression.

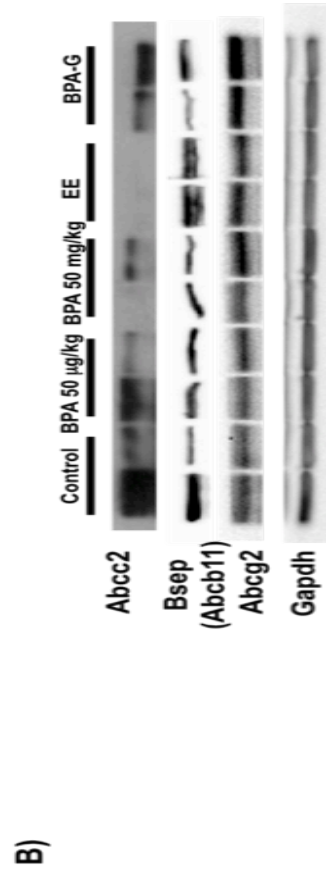
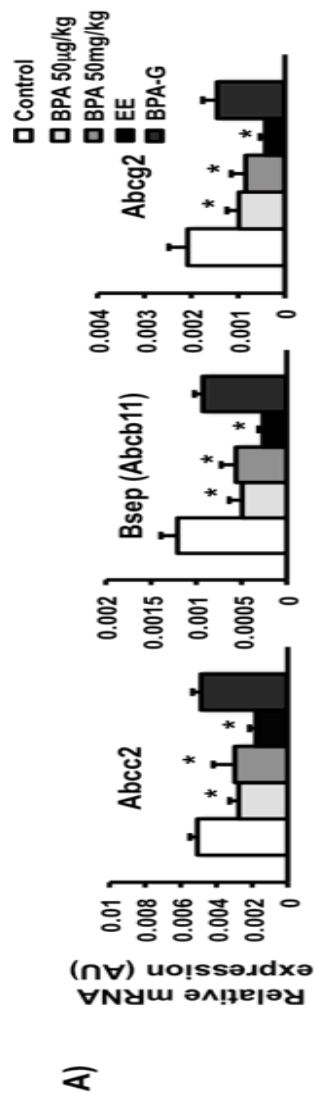


Figure 4. Effect of developmental BPA exposure on hepatic apical/canalicular transporter expression.

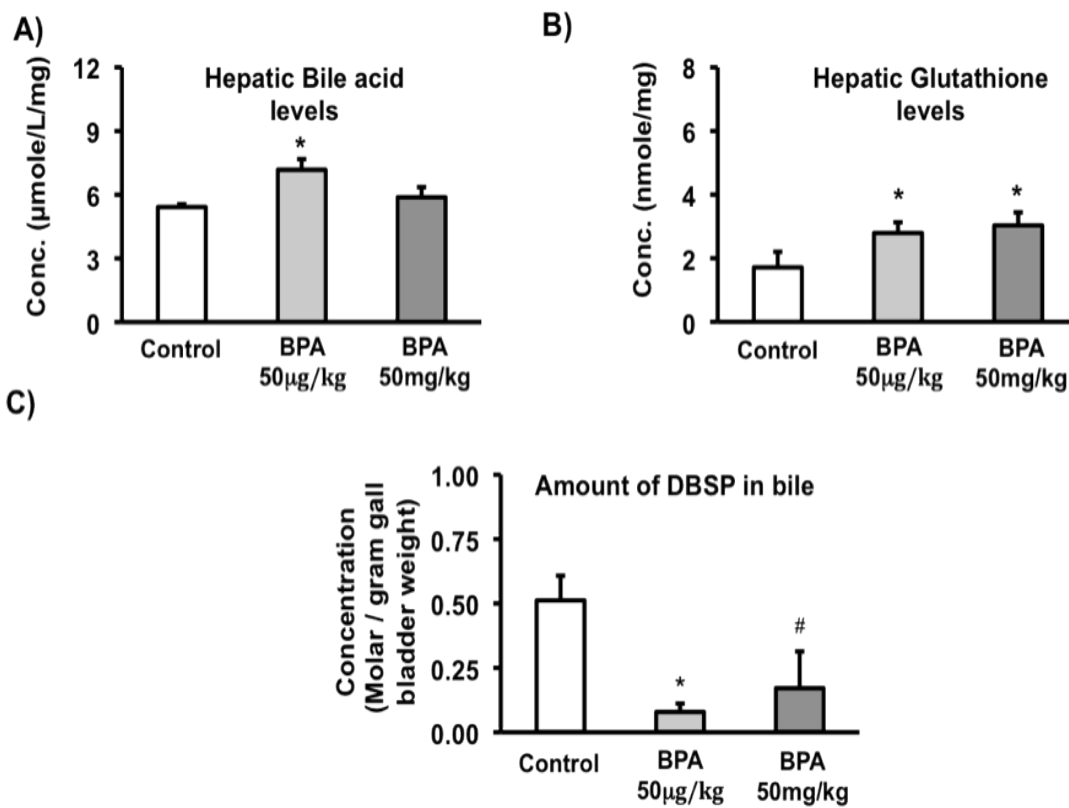


Figure 5. Effect of developmental BPA exposure on hepatic clearance.

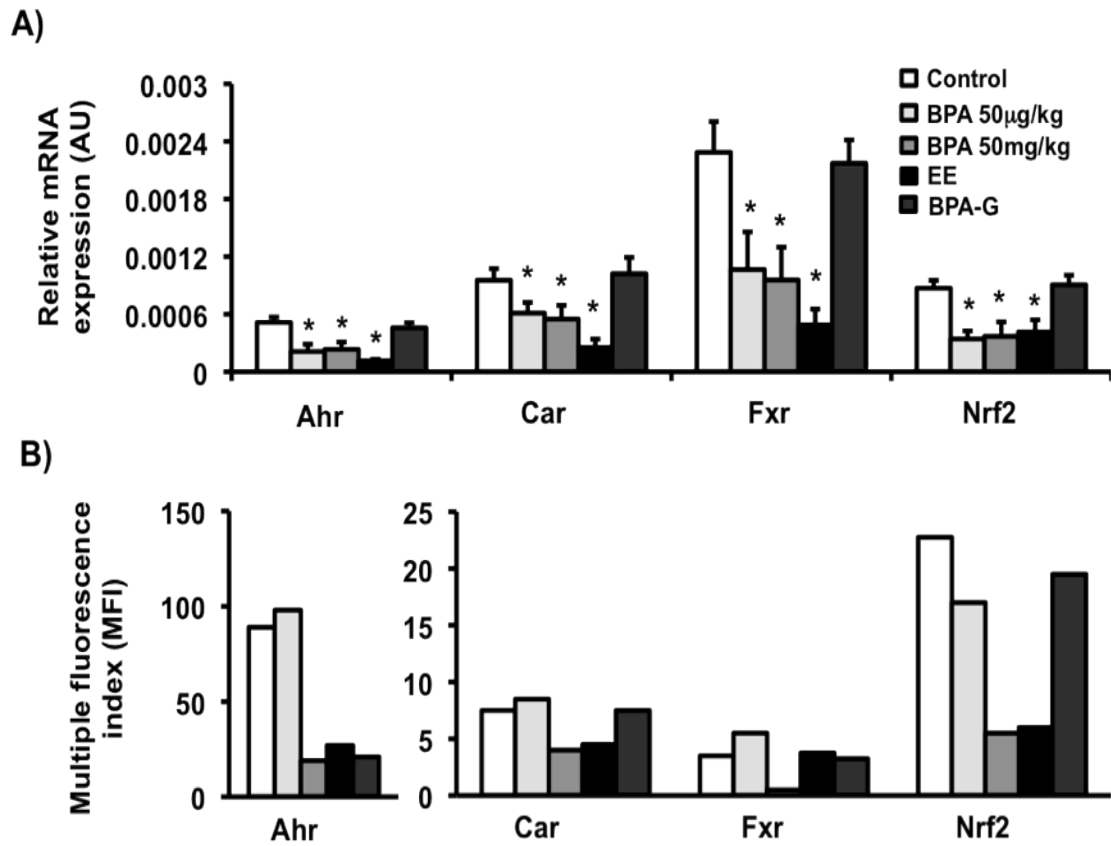


Figure 6. Effect of developmental BPA exposure on transcriptional factors expression and binding activity.

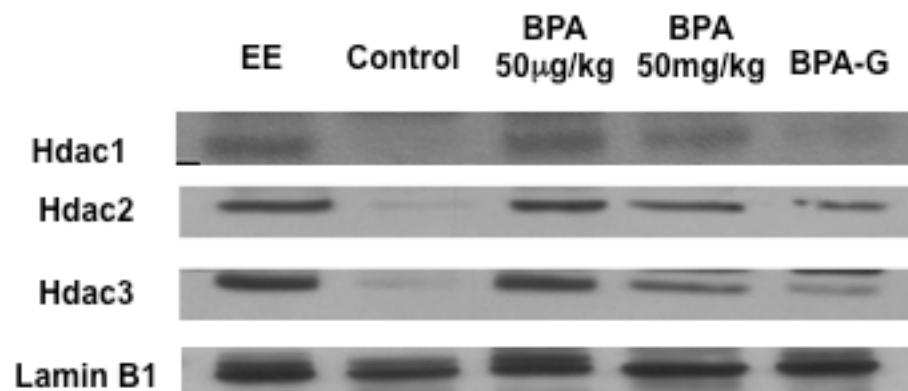


Figure 7. Effect of developmental exposure of BPA on nuclear levels of class I Hdac proteins.

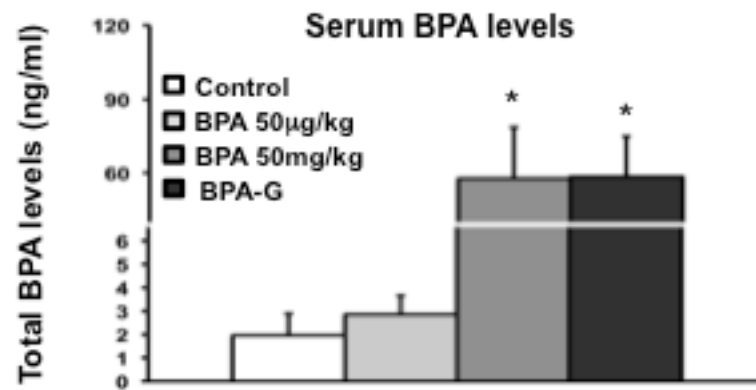


Figure 8. Serum BPA levels in dams exposed to different diets.

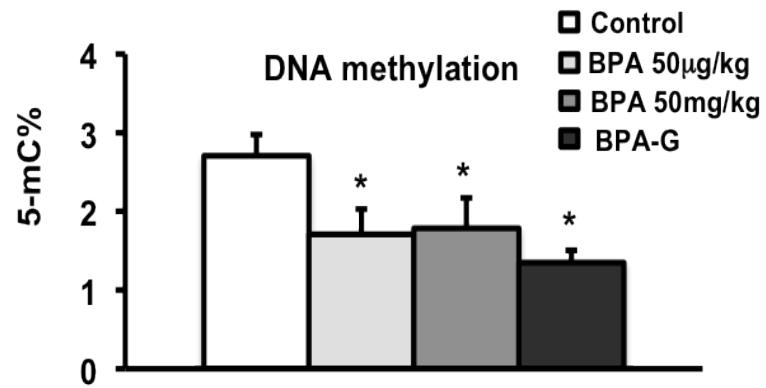


Figure 9. Effects of developmental BPA exposure on global methylation in male pups livers.

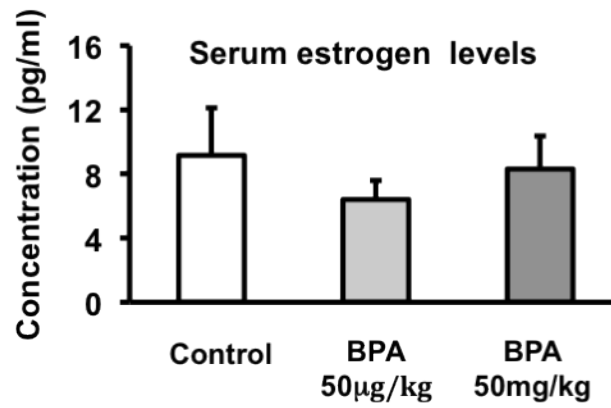


Figure 10. Effects of developmental BPA exposure on serum estrogen levels in male pups.

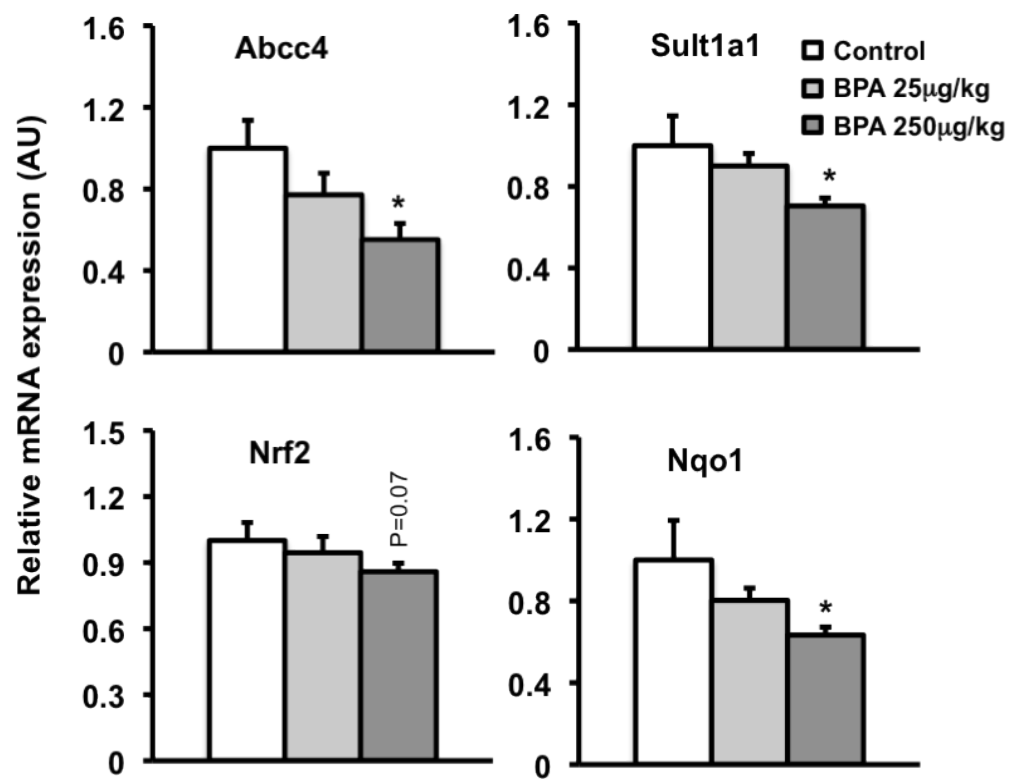


Figure 11. Effects of developmental BPA exposure on hepatic phase II and drug transporter expression in male CD-1 pups.

Table 1. Detailed sequences for list of primers used in qPCR for quantifying gene expression

Gene	Forward	Reverse
mAbbc2	AGCAGGTGTTTCGTTGTGTGT	AGCCAAGTGCATAGGTAGAGAAT
mAbcc3	CTGGGTCCCCTGCATCTAC	GCCGTCTTGAGCCTGGATAAC
mAbcc4	CCAGACCCTCGTTGAAAGAC	TGAAGCCCGATTCTCCCTTC
mAbcg2	GCGGAGGCAAGTCTTCGTTGC	TCTCTCACTGTCAGGGTGCCCA
Bsep (mAbcb11)	CCCATAAACATCAGCCAGTTGT	TCTGACTCAGTGATTCTTCGCA
Ntcp (mSlc10a1)	CAAACCTCAGAAGGACCAAACA	GTAGGAGGATTATTCCCGTTGTG
mUgt2b1	GTGCTGGTGTGGCCTACAG	ATTGCTCGGCCCAATGAGG
mSult1a1	AACATGGAGCCCTTGCGTAAA	ATGAGCACATCATCAGGGCAG
mAhr	AGCCGGTGCAGAAAACAGTAA	AGGCGGTCTAACTCTGTGTTC
mCar	ATATGGGCCGAGGAAGTGTGT	GGCGTGGAATGATAGCCTGT
mFxr	GCTTGATGTGCTACAAAAGCTG	CGTGGTGATGGTTGAATGTCC
mNrf2	TCTTGAGTAAGTCGAGAAGTGT	GTTGAAACTGAGCGAAAAAGGC
mNqo1	AGGATGGGAGGTACTCGAATC	AGGCGTCCTTCCTTATATGCTA
m18s	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA

MANUSCRIPT-III

Altered disposition of an endocrine disruptor- Bisphenol A (BPA) during obesity

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Key words: BPA dispositon, Obesity, MetS and NAFLD

ABSTRACT

Bisphenol A (BPA) is an endocrine disruptor available ubiquitously in environment. BPA is exposed to humans through several forms such plastic bottles, canned food and even through dental sealants. BPA is rapidly metabolized and excreted from body either through urine or feces. BPA glucuronide (BPA-Gluc) is a major metabolite whereas BPA sulfate (BPA-S) is minor metabolite formed. As BPA metabolites are termed non-toxic compounds, BPA metabolism has an important role in BPA induced toxicity. Studies have identified in humans, correlation between urinary BPA levels and occurrence of metabolic syndrome components such as obesity, diabetes and cardiovascular diseases. Obesity is a hallmark of metabolic syndrome, which is a complex condition involving accumulation of fat mass and changes in several gene expression. Both in humans and rodents, altered metabolism and disposition of xenobiotics were observed with obesity and other metabolic syndrome conditions. BPA disposition during metabolic syndrome conditions such as obesity, fatty liver and diabetes is very less known. In this study we administered BPA (10 mg/kg, *i.v.*) in lean and obese rats. Bile, blood and urine samples were collected at different time interval and analyzed using UPLC-MS/MS. Along with *in vivo* BPA disposition studies hepatic glucuronidation and sulfation enzymatic assays were performed to identify whether obesity altered hepatic metabolic processes. Our results indicate obese rats have decreased hepatic glucuronidation and increased hepatic sulfation. Changes in hepatic phase II and III protein expression in obese rats

resulted in altered BPA metabolism and disposition. In obese rats BPA metabolites specifically BPA-Gluc levels were increased in urine and decreased in bile compared to lean rats. These altered BPA metabolism and disposition during obesity suggests in humans, in depth evaluation of urinary BPA levels such as ratio of metabolite to parent compound are needed to correlate BPA exposure to occurrence of obesity.

INTRODUCTION

BPA is a bi-product of plastic manufacturing, available ubiquitously in environment and exposed to humans through several forms (Vandenberg et al., 2009). According National Health and Nutrition Examination Survey (NHANES) 92.6% of people analyzed have BPA in their urine (Calafat et al., 2008). Rodent studies documented, BPA can cause several adverse effects such as obesity, insulin resistance, oxidative stress, endocrine disruptors and behavioral changes (Somm et al., 2009; Vandenberg et al., 2009; Jasarevic et al., 2011). BPA is highly metabolized in liver and eliminated by urinary and fecal excretion (Pottenger et al., 2000; Volkel et al., 2002). BPA-glucuronide (BPA-Gluc) and BPA-sulfate (BPA-S) are the major metabolites formed in humans and mice. The UGT2B family and SULT enzymes are major biotransformation enzymes involved in BPA conjugation (Nishiyama et al., 2002; Hanioka et al., 2008). In rodents, Abcc2 is the predominant transporter, which mediates BPA-Gluc excretion from liver into bile (Inoue et al., 2005).

Obesity is a hallmark disease of metabolic syndrome and widely considered as a complex condition. Obesity is characterized by body mass index (BMI > 30 kg/m²). There is growing concerns of obesity affecting people worldwide and approximately above 30% of US population was effected by obesity of which 5% are considered as morbidly obese (Ogden et al., 2006; Flegal et al., 2010). The CDC estimates that more than 86% of US adults will be overweight and more than 50% obese by the year 2030 (Wang et al., 2008). One of the major complications during obesity is NAFLD, 15-39% of US population are

effected by NAFLD (Younossi et al., 2002; Collantes et al., 2004). Obese people have altered drug absorption, distribution, metabolism and elimination process. Several studies documented altered metabolism and drug disposition in both obesity and NAFLD disease conditions (Naik et al., 2013). During obesity altered pharmacokinetic parameter are related to several factors such as change volume of distribution, fat mass and clearance (Brill et al., 2012). Apart from these factors hepatic metabolism, which includes phase I, II and III (drug transporter) proteins also plays important role in pharmacokinetics aspects in obese population (Brill et al., 2012). Both in humans and rodents, altered phase I and, II enzymes and drug transporter expression were observed during NAFLD (Naik et al., 2013). Moreover, during obesity and non-alcoholic fatty liver disease (NAFLD) conditions phase II enzymes and drug transporters involved in BPA metabolism are altered (More and Slitt, 2011; Hardwick et al., 2013).

There is very less known about whether changes in hepatic metabolic pathways in obese population alters endocrine disruptors disposition. In this study we want to determine how obese population handle BPA body burden. In this study we also want to determine whether changes in phase II enzymes and drug transporters during metabolic syndrome conditions alter BPA metabolism and disposition. We used lean and obese zucker rat and injected BPA to identify how obese population handle high amount of BPA exposure.

Materials and Methods:

Animals: Nine-week old lean and obese zucker rats (n=6) were purchased from Charles River Laboratories International, Inc. (MA, USA). Rats were housed under a constant dark/light cycle (12 h/12 h), and given food and water *ad libitum* for 2 weeks at Department of Biomedical and Pharmaceutical Sciences vivarium. Rats used for this study are 11-week old. All the animal experiments were conducted at the University of Rhode Island using procedures approved by the Institutional Care and Use Committee (IACUC).

Surgical procedure: Both lean and obese zucker rats were anaesthetized using urethane (1.5 mg/kg, *ip*). Left femoral vein and artery were cannulated using PE-50 polyethylene tubing and bile duct was cannulated using PE-10 polyethylene tubing. Before injection of BPA femoral artery and vein cannula are flushed with saline. BPA (10 mg/kg, *i.v.*) (Moors et al., 2006) dissolved in polyethylene glycol 400 and isotonic saline (20:80, w/w) was injected through femoral vein. ~200 μ L of blood was collected from femoral artery into heparanized tubes at 0, 2, 10, 20, and 40 min time interval after injection of BPA. Equal amount of saline was injected through femoral vein after every blood collection. Bile samples were collected at 0, 15, 30, 45 and 60 min time intervals after BPA administration. At the end of 90 min, urine was collected using 3 mL syringe and 22-gauge needle by puncturing bladder (Lickteig et al., 2007). At the end rats were euthanized and liver were collected and snap frozen in liquid nitrogen. All samples collected were stored in -80°C until analysis.

Microsomes preparation: 200mg of liver tissue (n=3-6 per group) was washed with ice-cold saline and 50mM sodium pyrophosphate buffer (pH-7.4). Following washing livers were homogenized in 600 ml homogenizing buffer (0.25M Sucrose, 50mM Tris-Hcl (pH-7.8), 0.5mM EDTA, 20mM BHT, 0.1mM DTT) and centrifuged at 10,000x g for 15 min. Supernates were centrifuged again at 100,000x g for 60 min. Cytosolic supernate was transferred to tube and stored at -80°C for future use. Microsomal pellet was re-suspended in re-suspending buffer (0.25M Sucrose, 10mM Tris-Hcl (pH-7.8), 1mM EDTA, 20mM BHT and 20% glycerol) and stored at -80°C for future use.

Enzymatic Glucuronidation and analysis: Hepatic microsomal fractions (0.0075 mg) were incubated with 1 μ M BPA and 2mM UDPGA in 50mM phosphate buffer (pH-7.0) for 30 min at 37°C. At the end of Incubation, tubes were heated in boiling water to deactivate protein and centrifuged at 14000Xg for 10 min. Supernatant were collected and 50 μ L of each sample injected in to HPLC-UV (System Gold[®], Beckman Coulter, Inc.). BPA and BPA-Gluc were analyzed with a gradient elution, 80% ACN and 20% 20mM phosphate Buffer (pH-2.7) at 1 mL/min flow rate using ZORBAX SB-Aq column (5 μ m, 4.6X150mm, Agilent Technologies) (Wen *et al.*, 2013). BPA and BPA-Gluc were identified using standards of BPA (sigma-aldrich) and BPA-Gluc (Toronto research chemicals, Canada) made in 50 mM phosphate buffer (pH-7.0). BPA-Gluc and BPA are eluted at 7.4 and 10 minutes respectively. BPA and BPA-Gluc were quantified by comparing area of peak with standards.

Enzymatic Sulfation and analysis: The cytosolic fraction were incubated with 2.4 μ M of radiolabeled sulfonyl donor [35 S]-3'-phosphoadenosine-5'-phosphosulfate (35 S-PAPS) and 4 μ M BPA in 20 mM potassium phosphate (pH-7.0). The reaction mixture was incubated for a 60 min at 37°C, after incubation protein reaction was stopped by heating in boiling water for 30 sec, and centrifuged at 14,000 x g for 20 min to pellet the protein. For separation of reaction components, the resulting supernate was injected onto phenomenex synergi polar-RP column (50 x 2.00 mm, 4 micron). A linear gradient of 15-80% acetonitrile and 20 mM potassium phosphate (pH-2.7) in 8 min was used as mobile phase to separate excess 35 S-PAPS from BPA- 35 S. 35 S-labeled peaks were quantified on a flow scintillation analyzer (Packard Bioscience, 500 TR series) with Perkin-Elmer Ultima Flo-M scintillation cocktail. 35 S-PAPS was eluted with the solvent front and BPA- 35 S at 4.8 min.

Sample preparation and detection: BPA and BPA metabolites were detected using solid phase extraction followed by UPLC-MS/MS. Method used in this study is a modified version of previously published study by *Coughlin et al., 2011*. Briefly, 100 μ L of plasma, bile and urine samples were diluted to 1 mL with dilution buffer (Citrate, 100 μ L of 250 mM ammoniumacetate (pH 5), 80 μ L of 1 M formic acid, and water) and extracted using Bond Elut Plexa cartridges (Varian Inc., Palo Alto, CA, USA; 60 mg, 1 mL). BPA and its metabolites are eluted in 8mL of elution solution (ethanol, methanol and acetonitrile). Eluted solution is evaporated and extract was reconstituted in 200 μ L 75% acetonitrile in water. 10 μ L of reconstituted sample was injected

into UPLC MS/MS, BPA and its metabolites were quantified by comparing standards (Coughlin et al., 2011). In this study data represented from bile and urine sample have n= 6 samples per group whereas plasma samples are n= 5-6 per group.

Statistical Analysis: The statistical significance between groups was determined using one-tailed student t-test. Data are presented as mean \pm SE, with $P \leq 0.05$ considered statistically significant.

Results:

Enzymatic BPA glucuronidation and Sulfation: Figure 1 illustrates hepatic glucuronidation and sulfation in both lean and obese rats. Hepatic glucuronidation was significantly reduced in obese rats compared to lean rats by 42% (Figure 1A). *In Vitro* sulfation assay showed 1.4-fold increase in hepatic sulfation in obese Zucker rats compared to lean Zucker rats (Figure 1B).

Effect of obesity on BPA and its metabolite disposition: Cumulative biliary excretion of BPA and its metabolites are shown in figure 2. Obese Zucker rats have increased biliary excretion of BPA compared to lean rats (figure 2A). In obese rats, 21.82 $\mu\text{g/mL}$ of cumulative amount of BPA excreted into bile whereas lean rats have 7.85 $\mu\text{g/mL}$ indicating 3-fold increase of BPA in bile. BPA levels in bile are significantly higher at all time points in obese Zucker rats compared to lean rats. BPA-Gluc is a major metabolite quantitatively excreted into bile. In obese Zucker rats BPA-Gluc excretion into bile was significantly decreased compared to lean rats. Significant differences in BPA-Gluc levels in bile were observed at initial time points such as 15, 30 and 45 min. These significant differences in BPA-Gluc levels were diminished at later time points. Biliary excretion of BPA-S was decreased in obese compared to lean rats (figure 2C). Decrease in BPA-S biliary levels in obese rats was observed at all time points.

Figure 3 shows cumulative levels of BPA and its metabolites in plasma. Cumulative plasma BPA levels did not altered in obese rats compared to lean rats. Cumulative BPA-Gluc levels in plasma showed an increasing trend in obese compared to lean rats (figure 3B). Cumulative BPA-S levels in plasma were unaltered in obese rats (figure 3C). Figure 4 shows cumulative levels of BPA and its metabolites in urine. Interestingly, although no significant changes were observed with plasma BPA-Gluc and BPA-S levels urine BPA-Gluc and BPA-S levels increased significantly.

Discussion:

Etiology of obesity and metabolic syndrome includes several factors like diet, decreased physical activity, genetics and environmental chemicals (Heindel and vom Saal, 2009). Rodent studies identified several environmental chemical and drug molecules that can cause obesity and NAFLD (Heindel and vom Saal, 2009). Environmental chemicals that cause obesity are termed as obesogens (Heindel and vom Saal, 2009). Several studies have correlated urinary BPA levels in humans with altered kidney function and several condition of metabolic syndrome such as diabetes, cardiovascular and obesity (Lang et al., 2008; Melzer et al., 2010; Trasande et al., 2012; Trasande et al., 2013). Rodent studies identified both developmental and daily BPA exposure causes obesity and hepatic steatosis, a benign condition of NAFLD (Somm et al., 2009; Marmugi et al., 2012). As BPA exposure and occurrence of obesity are interlinked to each other, in this study we showed how changes in phase II and drug transporter expression during obesity alters BPA body burden.

Obese zucker rats have spontaneous mutation in *fa* gene (leptin receptor) present in chromosome 13 (Kanasaki and Koya, 2011). Obese zucker rats displayed hyperglycemia, hypelipidemia and hyperinsulinemia conditions and these rats are used as a rodent models for obesity, diabetes and NAFLD disease conditions (Kanasaki and Koya, 2011). As mentioned previously obesity and NAFLD disease condition have altered pharmacokinetics of drug substances in both human and rodents. Similar to obese patients, obese zucker rats displayed altered acetaminophen disposition and have decrease in

acetaminophen-induced toxicity (Blouin et al., 1987; Lickteig et al., 2007; Hardwick et al., 2013). Obese Zucker rats also displayed altered phase II enzyme and drug transporters expression. In this study we used obese zucker rats as a model to identify whether change in metabolism gene expression during obesity alters BPA disposition.

BPA majorly undergo extensive metabolism BPA-Gluc is a major metabolite formed whereas BPA-S is minor metabolite. In both humans and rodents BPA is metabolized extensively in liver and intestines (Pottenger et al., 2000; Volkel et al., 2002). In rodents, Ugt2b1 is a liver specific enzyme, which is predominantly involved in hepatic glucuronidation of BPA (Yokota et al., 1999). Studies have documented hepatic Ugt2b1 expression decreased in obese zucker compared to lean (Kim et al., 2004). Decrease in observed hepatic glucuronidation capacity in obese zucker rats is due to decrease in Ugt2b1 expression. In humans, BPA is metabolized by several UGT isoforms such as 2B15, 2B7, 1A8 and 1A9 (Hanioka et al., 2008). Among these isoforms UGT2B15 was considered to be major isoform involved in hepatic BPA glucuronidation (Hanioka et al., 2008). Interestingly in humans UGT isoforms expression involved in BPA metabolism were not altered with development of NAFLD, indicating BPA glucurondation may not be altered during obesity and NAFLD (Hardwick et al., 2013). Studies identified expression of certain UGT isoforms may not translate to their activity (Court, 2010). Lorazepam, and oxazepam, substrates for UGT2B15 were metabolized more in obese patients (Abernethy et al., 1983) whereas UGT2B7

substrate mycophenolic acid conjugation was decreased during diabetes (Dostalek et al., 2011). These changes indicate altered metabolism especially hepatic BPA glucuronidation during obesity, diabetes and NAFLD, which are components of metabolic syndrome.

Both in humans and rodents BPA sulfation is a minor pathway, which is catalyzed by SULT1A1 predominantly (Nishiyama et al., 2002). In rodents, diet induced obesity increased Sult1a1 expression, which is similar to SULT1A1 expression in human steatotic livers (Hardwick et al., 2013). SULT1A1 is involved in sulfation of phenolic compounds like acetaminophen. Studies have documented during NAFLD both in human and rodents acetaminophen sulfation increased (Lickteig et al., 2007; Hardwick et al., 2013). This indicates both humans and rodents have increased BPA sulfate formation during obesity.

In humans BPA is rapidly metabolized and excreted through urine whereas, in rodents BPA metabolites are excreted through feces (Volkel et al., 2002). Transporters involved in BPA metabolites excretion are different in human and in rodents (Mazur et al., 2012). BPA-Gluc, a major metabolite formed in BPA metabolism is transported by Abcc2 in rodents (Inoue et al., 2005). Transporter assays using membrane ATPase assays identified in humans ABCC3 might be involved in BPA-Gluc transport (Mazur et al., 2012). ABCC3 is efflux drug transporter present on basolateral membrane of hepatocyte and involved in excretion of endogenous and xenobiotics from liver into blood (Klaassen and Aleksunes, 2010). In obese Zucker rats hepatic Abcc2

expression was significantly downregulated (Geier et al., 2005). Decrease in hepatic phase II and III (drug transporter) expression in obese rats play major role in altered disposition of BPA. Decrease cumulative BPA-Gluc levels in bile is may be due to decreased Abcc2 expression. Studies have documented that rats lacking Abcc2 show decreased BPA-Gluc biliary excretion (Inoue et al., 2005). Decreased biliary BPA-Gluc excretion resulted in increase of plasma and urine cumulative BPA-Gluc levels.

Changes in humans, during NAFLD hepatic altered phase II and III protein expression was observed (Hardwick et al., 2011; More and Slitt, 2011; Hardwick et al., 2013). As mentioned previously, in humans during obesity increased glucuronide conjugate formation of lorazepam, and oxazepam was observed, which are substrates for UGT2B15 (Abernethy et al., 1983). Increase in UGT2B15 activity in obese people indicates that obese patient can detoxify BPA more efficiently than normal population. Moreover in humans, hepatic ABCC3 expression increased with increase in progression of NAFLD (Hardwick et al., 2011; More and Slitt, 2011). This indicates obese people will have high clearance of BPA-Gluc from liver, which ultimately results in increased BPA-Gluc urinary excretion.

As mentioned previously, in humans urinary BPA levels are correlated with obesity. In most of these studies only total urinary BPA levels were observed and these studies did not specified ratio of BPA and its metabolites in human urine samples (Carwile and Michels, 2011; Trasande et al., 2012; Wang et al., 2012). May be high amount of total BPA in obese human urine may be result

of altered metabolism and disposition of BPA. Correlation between obesity and urinary BPA levels observed from previous studies may need more evaluation. Detailed evaluation of BPA and its metabolites in human urine gives more information whether obesity and BPA exposure are interlinked in humans or not.

In conclusion, hepatic clearance plays a major role in detoxifying and elimination of BPA from body. We think changes in phase II and III protein during obesity will alter hepatic clearance of BPA and its metabolites. Our model of obesity in which altered hepatic phase II and III protein expression resulted in altered BPA metabolism and disposition. Although our model did not show same changes that are observed in obese human population, it strongly indicates that BPA metabolism and disposition will be altered during obesity. In humans, correlation between total BPA levels in urine cannot be correlated to obesity, as obese population is known to have altered ADME. In depth evaluation of BPA levels such as ratio of metabolite to parent compound are needed to correlate BPA exposure to occurrence of obesity.

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Figure Legends:

Figure 1. Altered hepatic BPA glucuronidation and sulfation due to obesity. A) BPA hepatic glucuronidation was decreased in obese zucker rats. Hepatic microsomes are incubated with 1 μ M of BPA and 1mM of UDPGA for 30 min at 37°C. Amount of BPA-Gluc formed was measured using HPLC-UV and data represented as average BPA-Gluc formed (nmol/min/mg) \pm S.E.M (n=6 per group). B) BPA hepatic sulfation was increased in obese zucker rats. Hepatic cytosolic fractions were incubated with 4 μ M BPA and 2.4 μ M 35 S-PAPS for 1 hour at 37 °C. Amount of BPA- 35 S formed was analyzed using HPLC attached to scintillation counter detector and data represented as average BPA- 35 S formed (pmol/min/mg) \pm S.E.M (n=6 per group). Asterisk (*) represents statistical significance between lean and obese zucker rats (p<0.05).

Figure 2. Altered hepatic BPA and BPA metabolites disposition in bile due to obesity. Cumulative biliary excretion of BPA (A), BPA-Gluc (B) and BPA-S (C) in both lean and obese zucker rats following BPA administration. Both lean and obese zucker femoral artery and, vein, and bile duct were cannulated. BPA (10mg/kg) administered through femoral vein and bile samples were collected at 15, 30, 45, 60 and 90 min time intervals. BPA and BPA metabolites were analyzed using UPLC-MS/MS technique. Asterisk (*) represents statistical significance between lean and obese zucker rats (p<0.05).

Figure 3. Altered hepatic BPA and BPA metabolites disposition in plasma due to obesity. Cumulative plasma levels of BPA (A), BPA-Gluc (B) and BPA-S (C) in both lean and obese zucker rats following BPA administration. Both lean and obese zucker femoral artery and, vein, and bile duct were cannulated. BPA (10mg/kg) administered through femoral vein and blood samples were collected at 2, 10, 20, 40 and 60 min time intervals. BPA and BPA metabolites were analyzed using UPLC-MS/MS technique. Asterisk (*) represents statistical significance between lean and obese zucker rats ($p < 0.05$).

Figure 4. Altered hepatic BPA and BPA metabolites disposition in urine due to obesity. Cumulative urinary excretion of BPA (A), BPA-Gluc (B) and BPA-S (C) in both lean and obese zucker rats following BPA administration. Both lean and obese zucker femoral artery and, vein, and bile duct were cannulated. BPA (10mg/kg) administered through femoral vein and urine samples were collected at 30, 60 and 90 min time intervals. BPA and BPA metabolites were analyzed using UPLC-MS/MS technique. Data represented as average of BPA or its metabolites (ng/mL) \pm S.E.M (n=6 per group). Asterisk (*) represents statistical significance between lean and obese zucker rats ($p < 0.05$).

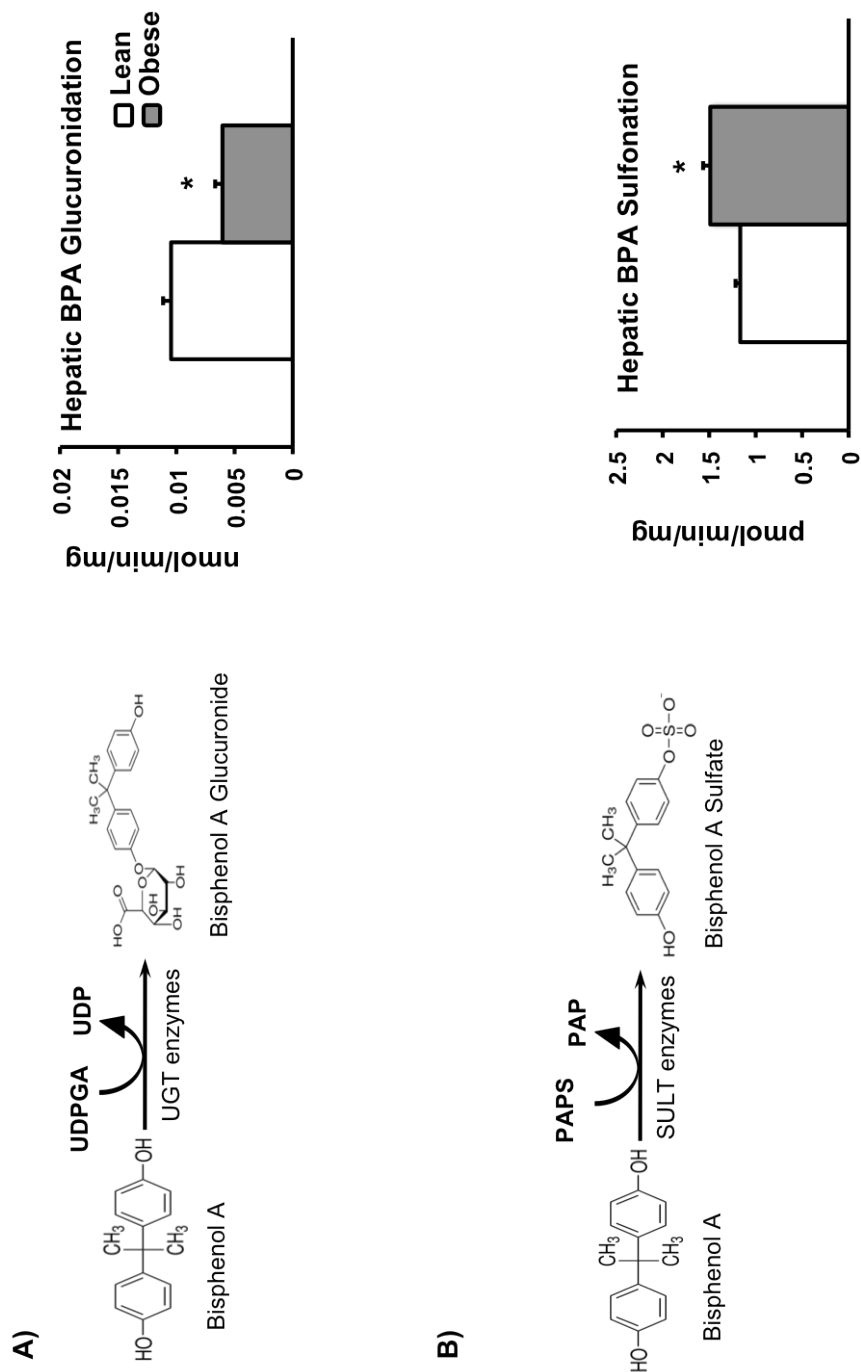


Figure 1. Altered hepatic BPA glucuronidation and sulfation due to obesity.

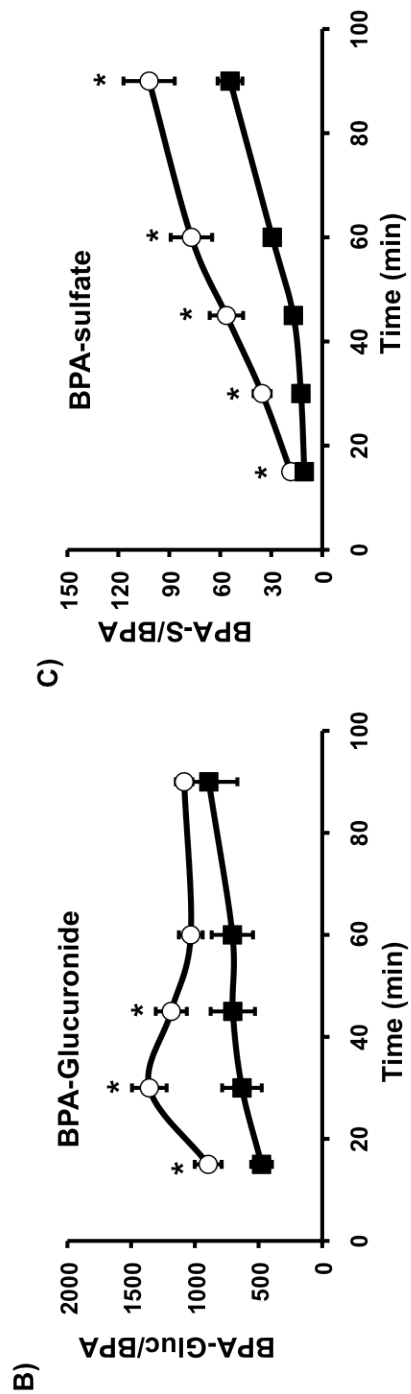
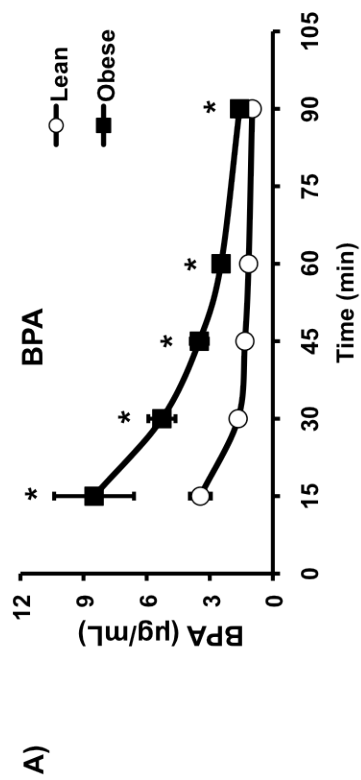


Figure 2. Altered BPA and BPA metabolites disposition in bile due to obesity.

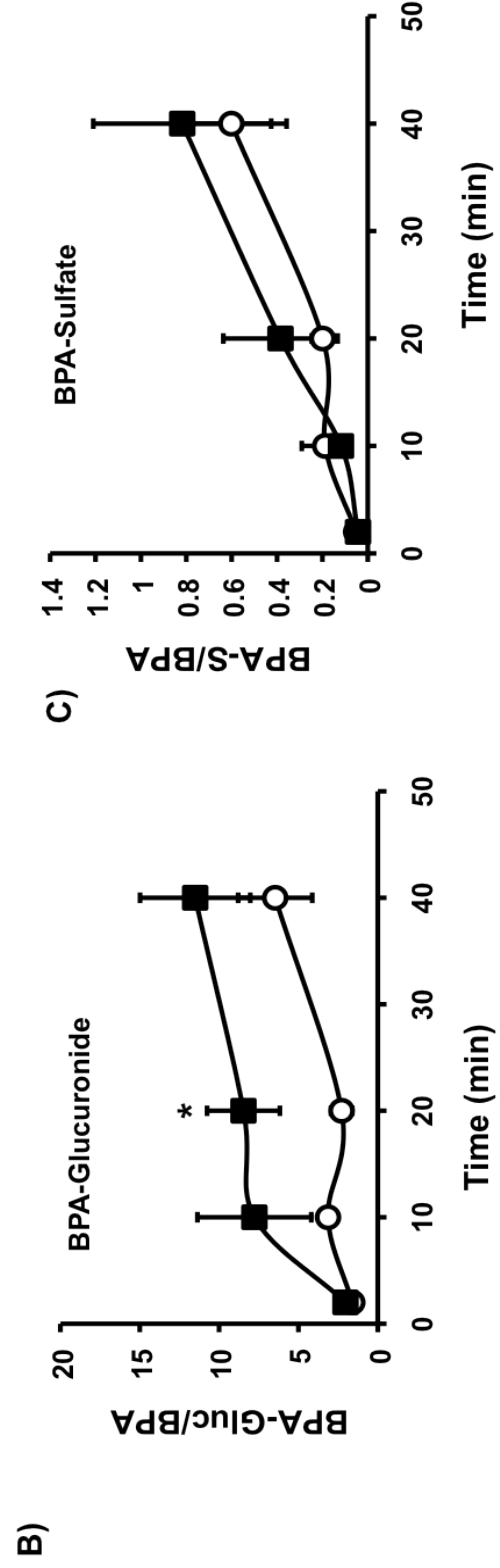
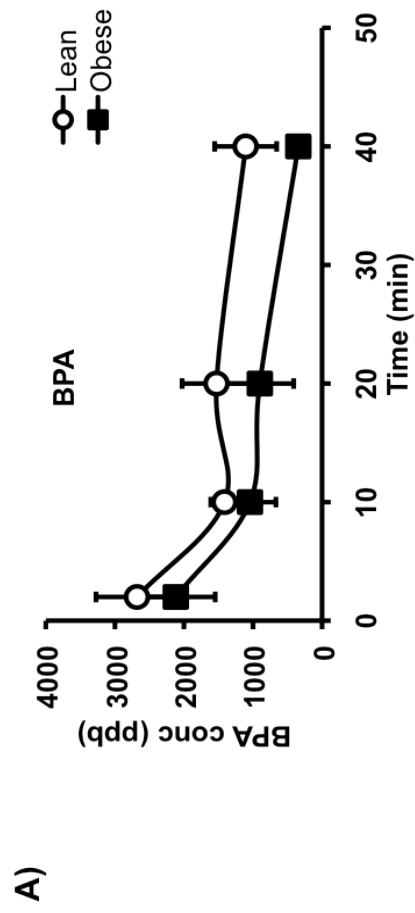


Figure 3. Altered BPA and BPA metabolites disposition in plasma due to obesity.

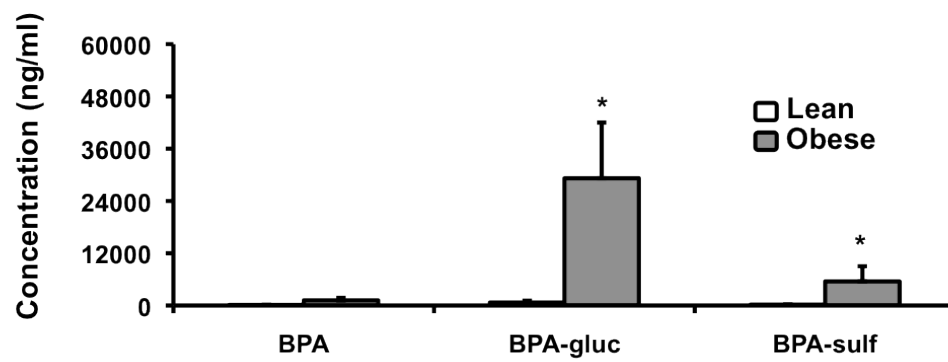


Figure 4. Altered BPA and BPA metabolites disposition in urine due to obesity.

Conclusion

In conclusion, we observed Abc drug transporter expression was altered with obesity, age and maternal diet. Changes in Abc drug transporters can alter hepatic clearance and endocrine disruptor disposition. Abc drug transporters expression was not only regulated by transcription factors but also with epigenetic mechanisms and serum metabolism-related hormones, which are involved with glucose and lipid metabolism. Detailed conclusions for each study are below:

In first study we observed ontogeny of drug transporters and physiological factors during development of obesity are sex specific. Sex-specific correlations were observed between physiological factors and gene expression. Sex-specific differences in correlations and expression of genes are may be due to regulation of hepatic drug transporters with endocrine hormones such as androgens, estrogen and growth hormones, which are known to be different in both male and females. Correlations between gene expression and physiological factors indicate serum hormone may have a role in regulation in drug transporters during development of obesity. In males, high correlations with serum resistin and glucagon with hepatic Abc transporter expression indicates serum resistin and glucagon can be a possible biomarker for change in Abc drug transporter expression during development of obesity.

Second study illustrated that maternal diet can regulate hepatic gene expression. In male pups, BPA developmental exposure decreased key hepatic Abc drug

transporters, which are involved in efflux of xenobiotics and endogenous hormone conjugates from liver. Observed developmental BPA exposure effects are similar to estrogen developmental exposure effects. BPA and estradiol development exposure also decreased transcription expression and activity, which were involved in regulation of Abc expression in liver. Phytoestrogen such as genistein supplementation in maternal diet along with developmental BPA exposure reversed changes observed with BPA exposure. Global decrease in hepatic Abc drug transporters, Phase II enzyme and transcription factor expression and activity caused by BPA and estradiol involves epigenetic effects. Both BPA and estradiol developmental exposures increased histone deacetylases (Hdacs) expression whereas genistein co-administration decreased Hdacs, which are involved in down regulation of gene expression by epigenetic mechanisms.

In third study we observed altered disposition of BPA during obesity. Changes in hepatic Phase II enzymes and Abc drug transporters are reflected in changes in BPA disposition. Specifically, decrease in hepatic UDP-glucuronosyl transferases 2b1 (Ugt2b1) in obese Zucker rats resulted in decrease in BPA glucuronidation in obese rats. Decrease in BPA glucuronidation, which is a major pathway for detoxification of BPA, increases body burden of BPA in obese rats. Decrease in hepatic Abcc2 expression obese rats resulted decrease in BPA-glucuronide (BPA-gluc) levels in bile which eventually resulted in increase of BPA-gluc levels in plasma and urine. Increase of hepatic sulfonation in obese rats caused increase in BPA-S levels in urine, although BPA-S decreased in bile.

These results supports that altered Phase II and Abc drug transporter expression in liver during obesity will results in altered disposition of endocrine disruptors and increases body burden of BPA.